

**From the Immunological Research Laboratory
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**Circulating Immune Complexes
in Atherosclerotic Vascular Disease**

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*To
My family
My father
The memory of my mother*

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LIST OF PAPERS

This thesis is based on the following papers, which are referred to by their roman numerals:

- I** **Mustafa A**, Nityanand S, Berglund L, Lithell H, Lefvert AK. Circulating immune complexes in 50-year-old men as a strong and independent risk factor for myocardial infarction. *Circulation* 2000; 102:2576-2581.
- II** **Mustafa A**, Ruotolo G, Ericsson CG, Nilsson J, de Faire U, Hamsten A, Lefvert AK. Role of circulating immune complexes and autoantibodies against modified LDL and cardiolipin in the progression of premature coronary atherosclerosis. Submitted.
- III** **Mustafa A**, Hamsten A, Holm G, Lefvert AK. Circulating immune complexes induced by food proteins implicated in precocious myocardial infarction. *Annals of Medicine* 2001; 33:103-112.
- IV** Nityanand S, Truedsson L, **Mustafa A**, Bergmark C, Lefvert AK. Circulating immune complexes and complement C4 null alleles in patients operated for premature atherosclerotic peripheral vascular disease. *Journal of Clinical Immunology* 1999; 19:406-413.
- V** Ahmed E, Nityanand S, **Mustafa A**, Brismar K, Lefvert AK. Anti-cardiolipin antibodies and circulating immune complexes in type 1 Diabetes Mellitus: Increased prevalence and relation to vascular complications. *Clinical and Experimental Immunology* 1999; 115:255-259.

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LIST OF ABBREVIATIONS

aCL	Anticardiolipin antibody
aPL	Antiphospholipid antibodies
APS	Anti-Phospholipid Syndrome
apo B	Apolipoprotein B
BSA	Bovine serum albumin
β2-GPI	β2-Glycoprotein I
C1q	Complement factor 1q
C3b	Complement factor 3b
C4	Complement factor 4
C4Q0	Null alleles of C4
C4A*Q0	Null alleles of C4A
C4B*Q0	Null alleles of C4B
CAD	Coronary artery disease
CHD	Coronary heart disease
CICs	Circulating immune complexes
<i>C. pneumoniae</i>	<i>Chlamydia pneumoniae</i>
CR1	Complement receptor type 1
ELISA	Enzyme-linked immunosorbent assay
ECM	Extracellular matrix
HDL	High density lipoproteins
ICs	Immune complexes
IDDM	Insulin dependent diabetes mellitus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDL	Low density lipoproteins
MAC	Membrane attack complex (C5b-9)
MDA-LDL	Malondialdehyde modified low density lipoprotein
MI	Myocardial infarction
OxLDL	Oxidatively modified low density lipoproteins
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SD	Standard deviation
TG	Triglycerides
VLDL	Very low density lipoprotein
vWFAg	von Willebrand factor antigen

INTRODUCTION

Atherosclerosis was once thought to be a degenerative disease and an inevitable consequence of ageing. Recent research has shown that it is neither degenerative nor inevitable. Atherosclerosis is best described as a chronic inflammatory disease caused by specific cellular and humoral immune responses (Ross 1993; Ross 1999; Ross and Glomset 1973). The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries.

The earliest recognizable lesions of atherosclerosis, the so-called fatty streaks, are common in infants and young children (Napoli et al. 1997). This lesion consists of an aggregation of lipid-rich macrophages and T lymphocytes (Sary et al. 1994). Small fibrofatty plaques are common in young adults and extensive atheroma lesions from early middle age on. These lesions increase in number with age.

Despite the knowledge of many risk factors for atherosclerosis, its complications, such as coronary heart disease (CHD) remain the leading cause of death in many developed nations, including the United States and most European nations. In a follow-up (through 1992) of the first U.S. National Health and Nutrition Examination Survey (1971-1975), having four or five risk factors increased the risk for a CHD event by five times and risk for death by three times (Yusuf et al. 1998). Contrary to old believes the rate is also increasing alarmingly even in developing countries.

This is why there is a great interest in the pathogenic mechanisms of atherosclerosis and its clinical complications. Epidemiological studies have identified several risk factors for atherosclerosis (Table 1). These classical risk factors explain only a part of the epidemiological features of atherosclerotic diseases. Thus, there are likely other factors that

increase the risk of atherosclerosis alone or in combination with the classical risk factors.

Table 1. Classical risk factors for atherosclerosis

Modifiable risk factors	Nonmodifiable risk factors
Elevated levels of LDL and VLDL	Age
Low levels of HDL	Male gender
Hypertension	Family history of premature atherosclerosis
Diabetes mellitus	
Cigarette smoking	
Infections	
Elevated levels of homocysteine	
Elevated levels of hemostatic factors, e.g. fibrinogen	
Insulin resistance	
Diet	
Obesity	
Physical inactivity	
Emotional factors	

Additional risk factors for atherosclerosis have been suggested to be circulating immune complexes (CICs) and autoantibodies against cardiolipin (aCL) or other negatively charged phospholipids, oxidatively modified low density lipoproteins (oxLDL), heat shock protein 60/65 and β 2GPI.

Pathogenesis of atherosclerosis

The normal and atherosclerotic vessel wall

The walls of arteries are composed of three well-defined layers called tunics. The layers, from the lumen outward, are tunica intima, tunica media and tunica adventitia. The intima is composed of a single layer of endothelial cells seated on a specialized extracellular matrix (ECM), the basement membrane. The media contains densely packed smooth muscle cells (SMC) arranged in single or multiple layers, surrounded by an ECM of collagen types I and III, fibronectin and chondroitin/dermatan sulphate

proteoglycans. The adventitia consists of fibroblasts, SMC, the vasa vasorum and the nerves, all placed in a loose layer of thick bundles of collagen (predominantly type I) and elastic fibres (Wight 1995; Wight 1996).

The atherosclerotic lesion is the most common acquired abnormality of blood vessels. The exact mechanism of atherogenesis is still unclear though there are three components, cholesterol, infection and inflammation enrolled in the genesis of atherosclerosis (Pentikäinen et al. 2000). We appreciate today the importance of these components but we are still ignorant of their interrelations, and do not know which of the three comes first. The pathological processes of atherosclerosis are thought to be initiated by interaction between modified forms of LDL, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall (Glass and Witztum 2001). The recruitment and migration of monocytes and T cells to the arterial intima from blood stream to the vessel wall is believed to be the initial step in atherosclerosis and largely governed by adhesion molecules and chemokines (Hansson and Libby 1996). These inflammatory processes can ultimately lead to the development of complex lesions, or plaques, within the innermost layer of an artery wall. These plaques protrude into the arterial lumen, narrow the vessel lumen and reduce the flow of blood to the tissue/organ it supplies. Plaques that are likely to rupture and stimulate thrombogenesis are termed unstable. Evidence suggest that enzymes released from macrophages eat away at the thin, lipid-rich and foam cell-rich peripheral margins of the plaque, precipitating plaque rupture, which in turn leads to thrombosis (Fuster et al. 1992) and results in the acute clinical complication of myocardial infarction (MI) or stroke (Navab et al. 1996; Ross 1999; Steinberg and Witztum 1999).

Vascular injury

Numerous pathophysiologic observations in humans and animals led to the formulation of the response-to injury hypothesis of atherosclerosis. This hypothesis initially proposed that endothelial denudation or dysfunction was the first step in atherosclerosis. The hypothesis states that the protective, inflammatory response followed by the formation of a fibroproliferative response with time and continuing insult may become excessive. Thus, both the inflammation and the fibrous connective tissue proliferation become, in themselves, the disease process (Ross 1999). Possible causes of endothelial dysfunction include elevated and modified LDL; ICs; free radicals caused by cigarette smoking, hypertension, and diabetes mellitus; elevated plasma homocysteine concentrations; infectious microorganisms; and combinations of these factors.

Mast cells and neutrophils

Mast cell and neutrophils have also been proposed to play a role in the disease progression. In the coronary artery, the majority of mast cells are found in the outer layer of adventitia (ten times as many as in the intima) (Stary 1990). Mast cells were recently shown to have accumulated in coronary plaques (Kovanen et al. 1995). Activated mast cells, found in the shoulder region and adventitia of advanced and complicated plaques, secrete enzymes such as neural protease and histamine, which may degrade extracellular matrix, cause rupture of atheromatous plaque and induce acute coronary syndrome (Kaartinen et al. 1994; Kaartinen et al. 1998; Kovanen et al. 1995).

Furthermore, stimulated mast cells release other vasoactive substances, such as prostaglandin D₂ and leukotriene C₄ that may mediate coronary spasm. Indeed, in atherosclerotic coronary arteries, histamine is a

powerful vasoconstrictor (Ginsburg et al. 1984) and the concentration of histamine in the coronary circulation was found to be elevated shortly before coronary spasm in patients suffering from variant angina. It has been shown that histamine released from the degranulated mast cells may reach the media, where it may locally provoke coronary spasm and thus contribute to the onset of MI (Laine et al. 1999). Activation of neutrophils is also detected in patients with unstable angina (Mehta et al. 1989). Neutrophils may have similar roles as mast cells to contribute to the destabilization of the plaques since they release elastase and oxygen radicals that can lead to degradation of the plaque connective tissue (de Servi et al. 1991; Dinerman et al. 1990). Neutrophils have however, not been detected in the lesions.

Infections

Infections are known to increase the blood viscosity, cause hypercoagulability, and influence the serum lipid profile. Seroepidemiological studies have detected a correlation between the incidence of atherosclerotic complications and the presence of infectious microorganisms, *Chlamydia pneumoniae* (*C. pneumoniae*) (Grayston et al. 1993; Linnanmäki et al. 1993; Saikku 1997; Saikku et al. 1988), *Helicobacter pylori* (*H. pylori*) and cytomegalovirus (Danesh et al. 1997). *C. pneumoniae* is a common pathogen in respiratory infections and has been detected in atherosclerotic lesions (Kuo et al. 1993). It can survive intracellularly in macrophages, which could be important for transportation of *C. pneumoniae* in the human organism (Gieffers et al. 2001). It has been proposed that *C. pneumoniae* aggravates atherosclerosis by activating macrophages to secrete tumor necrosis factor and metalloproteinases (Kol et al. 1998) and/or by eliciting production of antibodies that cause endothelial cytotoxicity (Mayr et al.

1999). In addition, it has been shown that molecular mimicry between proteins of *C. pneumoniae* and structural proteins of the myocardium can cause autoimmune myocarditis. This mechanism may also result in heart disease in infected individuals (Bachmaier et al. 1999). The presence in the lesions of modified or cross-reactive antigens that are capable of mounting an autoimmune response supports the autoimmune concept of atherosclerosis. Furthermore, antigens maintaining the inflammatory process in the arterial wall must be ubiquitous and be present at young age, explaining the overall prevalence of atherosclerosis even in young people (Stary 1989). However, there are some discordant reports on this subject. No direct evidence has been provided to establish a causative role for *H. pylori* (Folsom et al. 1998) and cytomegalovirus (Kol et al. 1995). In a recent study, infection with *C. pneumoniae* in mice did not induce atherosclerotic lesions (Caligiuri et al. 2001). Thus, to resolve this discrepancy further studies will be necessary to clarify the association between infectious microorganisms and atherosclerosis in humans.

Autoimmunity in atherosclerosis

CICs

Much interest has been focused on the putative role of autoantibodies and CICs. The term IC denotes a reaction product resulting from the interaction between an antigen and antigen-specific or nonspecific antibody and is used synonymously with antigen-antibody complex. Immune complex formation is a highly efficient mechanism of host defense directed to eliminate the antigen and favor an adequate immune response. Both autoantigens and exogenous antigens can trigger the formation of ICs. If the antigens are foreign, these processes usually

benefit the host leading to removal of antigens; if removal fails or they are autoantigens, immune complex formation may become detrimental. Antigens proposed or shown to elicit antibody responses related to atherosclerosis are endothelial surface antigen (Cerilli et al. 1985), sperm components (Clarkson and Alexander 1980), microorganisms (Benditt et al. 1983), lipoproteins (Beaumont and Beaumont 1977; Ho et al. 1976), glycosylated lipoproteins (Witztum et al. 1984), cardiolipin (Hamsten et al. 1986), oxidized lipids (Salonen et al. 1992), structural vessel wall antigens (Gerö et al. 1975; Gudbrandsson et al. 1981) and milk proteins (Annand 1986; Davies et al. 1969). That ICs were possible pathogenic factors in arteriosclerosis was initially suggested by data published by Minick & Murphy, who showed that the induction of chronic serum sickness in rabbits fed a lipid-rich diet resulted in the formation of vascular lesions similar to those seen in human arteriosclerosis (Minick and Murphy 1973). It is clear today, that CICs accelerate atherosclerosis in experimental animals (Wissler et al. 1989). Repeated immunisation of mice with protein antigens leads to the formation of CICs, which in turn causes inflammation of the vessel wall and acceleration of the diet-induced atherosclerotic process (Qiao et al. 1993). The presence of CICs in patients with CAD has been described in several independent investigations (Farrell et al. 1977; Füst et al. 1978; Lefvert et al. 1995; Lopes-Virella et al. 1999; Smith et al. 1983; Versey and Gabriel 1974). Persistence of CICs is however more rare (Steffen et al. 1986). The constituents of the CICs in post-infarction patients are mostly unknown. LDL-containing ICs have been demonstrated in patients with vascular disease and in healthy subjects (Klimov et al. 1985; Salonen et al. 1992; Vaarala et al. 1993). The immunoglobulins isolated from vascular lesions of Watanabe heritable hyperlipidemic (WHHL) rabbits and humans recognize oxLDL and are present in lesions as ICs with oxLDL (Ylä-

Herttuala et al. 1994). Such ICs can significantly perturb lipoprotein and cholesterol metabolism when incubated with human monocyte-derived macrophages (Gissinger et al. 1991; Griffith et al. 1988; Lopes-Virella et al. 1991).

ICs are found in the circulation and/or other biological fluids of patients with a wide spectrum of diseases, such as autoimmune, infectious and neoplastic disorders (Table 2).

Table 2. Diseases associated with immune complexes

- **Autoimmune diseases**
RA, SLE, Sjögren's syndrome, periarteritis nodosa, systemic sclerosis, mixed connective tissue disease, Felty's syndrome.
 - **Glomerulonephritis**
Exogenous and endogenous antigens
 - **Neoplastic diseases**
Solid and lymphoid tumors
 - **Infectious diseases**
Bacterial: Infective endocarditis, meningococcal infections, disseminated gonorrheal infection, infected ventriculoarterial shunts, streptococcal infection, leprosy, syphilis
Viral: Dengue hemorrhagic fever, cytomegalovirus infection, viral hepatitis B and C, infectious mononucleosis, subacute sclerosing panencephalitis
Parasitic: Malaria, trypanosomiasis, schistosomiasis, filariasis, toxoplasmosis
 - **Other conditions:**
Dermatitis herpetiformis and celiac disease, ulcerative colitis and Crohn's disease, MI, idiopathic interstitial pneumonia, pulmonary eosinophilic granuloma, cystic fibrosis, saccoidosis, multiple sclerosis, myasthenia gravis, uveitis, otitis media, sickle cell anemia, idiopathic thrombocytopenic purpura, primary biliary cirrhosis, kidney and bone marrow transplantation, pregnancy, preeclamptic and eclamptic syndrome, Lyme arthritis, steroid-responsive nephrotic syndrome, xanthomatosis, vasectomy, oral ulceration and Behçets syndrome, pemphigus and bullous pemphigoid, IgA deficiency, thyroid disorders, ankylosing spondylitis, diabetes, Henoch Schönlein purpura, cutaneous vasculitis.
-

Biology of immune complexes

ICs are three-dimensional structures forming a lattice between antigens and antibodies. There are two main types of reactions responsible for the antigen-antibody lattice: antigen-antibody bonds and Fc-Fc interactions.

Antigens are mostly multivalent i.e. they have multiple antigenic sites, and antibodies have at least two combining sites. Thus, it is possible to have an infinite structure of alternating antigen-antibody bonds. It is now known that the rapid precipitation reaction observed in many antigen-antibody systems cannot be explained adequately by the infinite-lattice theory. Experimental data demonstrate that such immediate precipitation is due to Fc interactions. Thus, ICs made with F(ab)₂ fragments from IgG precipitate more slowly than complexes made with whole IgG (Møller 1979; Møller and Steensgaard 1979).

Fc-Fc interactions explain the immediate aggregation of complexes in states of antibody excess, at equivalence and even in states of antigen excess. Such interactions facilitate close contact between antigen and antibody and may allow molecular rearrangements that favor the more stable infinite lattice to take place.

There are three possible sites of antigen-antibody interaction and IC formation:

- When structural antigens making up part of the surface of cells or fixed intercellular structures react with antibody, the ICs are essentially fixed at the site of the structural antigen, and whatever inflammation or disease results from such complexes is localized to a very specific site. Goodpasture's syndrome in man is a clinical example.
- When soluble ICs form from secreted or injected soluble antigens reacting with antibodies near the site of release or injection, the injury induced by these ICs tend to be localized. The experimental example of this form of IC injury is the Arthus reaction, and the clinical one is thyroiditis in man, in which antibodies to thyroid hormones are formed.

- Finally, when soluble antigens in circulation react with antibodies, CICs are formed. Such complexes may be trapped in one or more of the vascular or filtering structures of the body. They are without immunological relationship to their sites of deposits. The classic experimental model of systemic immune complex disease is serum sickness. SLE, glomerulonephritides, vasculitides and rheumatoid arthritis are human immune complex-mediated diseases.

Role of complement in clearance of ICs

Two distinct pathways activate the complement cascade, the classical and the alternative pathways (Lachmann and Peters 1982). The classical pathway is initiated by the binding of C1 molecule to the C1q-binding site in the C_H2 domain of antibody molecules (IgG1, IgG2, IgG3 or IgM) that have reacted with an antigen. The alternative pathway is less selective and initiated by microorganisms and by immunoglobulins (IgG, IgA, IgE) in complexes (Kuby 1994). The major biological function of complement is opsonization, the coating of antigen-antibody complexes and of particulate activators of the alternative pathway with large number of C3b molecules. Opsonization facilitates the clearance of ICs from circulation by binding to receptors specific for C3b (CR1) on the surfaces of various cells. About 95% of CR1 in the circulation in humans are present on erythrocytes. CICs bound to CR1 are transported to the liver and spleen and removed by fixed macrophages. This process prevents the formation of large lattices and immune precipitation. It is now clear that complement can substantially modify Fc-Fc interactions (Schifferli et al. 1986). Thus complement is capable to solubilize a preformed immune precipitate (Miller and Nussenzweig 1975). CR1 can also inhibit full activation of the complement cascade.

Two observations of biologic relevance are worth noting:

- Complement can inhibit the immune aggregation of about 10 times the quantity of ICs that it can solubilize, a reflection of the relative ease of preventing Fc-Fc interactions responsible for the immediate aggregation reaction, as compared with inducing disruption of the lattice.
- The two reactions differ in their inflammatory potential, since inhibition of immune precipitation generates less C3a and C5a, called *anaphylatoxins*, than does solubilization.

Complement C4A and C4B

The fourth complement factor (C4), a three-chain glycoprotein of about 200 kDa molecular weight, is a major protein of the classical pathway of complement activation. C4 plays a central role in modulating the reaction sequence leading to C3 activation (Kuby 1994). The cleavage of C4 by the activated C1 complex leads to the formation of two fragments: a small fragment (C4a) anaphylatoxin and an activated large fragment (C4b). Of particular importance is the covalent binding of the nascent C4b fragment to antibody-coated cells, antigen-antibody complexes and microorganisms (Law et al. 1984). C4 exists as two isotypes, C4A and C4B encoded by separate genes located only 10 kilobases apart within the major histocompatibility complex (MHC) class III region on chromosome 6 (O'Neill et al. 1978; Rittner et al. 1975). The MHC class III region also includes genes for C2 and factor B. Both C4 genes are polymorphic with at least 35 alleles, including null alleles at both loci (Hauptmann et al. 1988). The two isotypes C4A and C4B are similar and only a sequence of 4 amino acids in the C4d region located in the α chain is responsible for the major structural and functional differences. C4A products bind more effectively to amino groups as on protein-antigen

complexes, whereas C4B products bind more efficiently to hydroxyl groups and thus to carbohydrate cell surfaces as on microorganisms (Isenman and Young 1986; Law et al. 1984). Thus, the two isotypes are complementary, each showing preference for different sites on acceptors. Complete deficiency of C4 is a rare condition but a relatively high frequency of null alleles of C4A (C4A*Q0) of 5-15% and of C4B (C4B*Q0) of 10-20% are found in the normal population. These null alleles are defined by the heterozygous or homozygous lack of C4A or C4B proteins in the plasma. C4A*Q0 are often associated with autoimmune diseases especially those characterised by formation of ICs. Several studies have shown that SLE patients have an increased prevalence of C4A*Q0. The majority of the cases of complete C4 deficiency presented with SLE and SLE-like diseases. Homozygous C4B deficiency has been reported in IDDM, especially in cases of early onset disease related to virus infection. As the C4B isotype is able to bind more efficiently to membrane surfaces, a reduction in the concentration of this isotype may impair the capacity to eliminate foreign pathogens. This would also explain the observation of increased susceptibility to infection in C4B deficiency (Hauptmann et al. 1988).

Autoantibodies to modified LDL

Lipoproteins, including chylomicrons, VLDL, IDL, LDL and HDL are spherical complexes. They consist of a coat of apolipoproteins, amphiphilic phospholipids, unesterified cholesterol, and a core of cholesterol-ester and triglycerides. The main biological function of LDL is to carry serum cholesterol from blood into tissue (Brown and Goldstein 1986). After crossing the capillary endothelium, some LDL bind to LDL-receptors on extrahepatic tissue cells via a special sequence of the apoB-100 (Brown et al. 1981). The highest concentration of LDL receptors

occurs in the liver. This receptor-mediated uptake is tightly regulated: once the cell has obtained the cholesterol it needs, it shuts down LDL receptor synthesis and so prevents intracellular accumulation of cholesterol (Brown, Goldstein 1986). Any excess of LDL particles not taken up by cells is rapidly removed by the lymphatic system, which prevents extracellular accumulation of LDL particles. The fate of LDL particles that enter the arterial intima differs from that in other extrahepatic tissues because the arterial intima is a tissue without blood or lymphatic capillaries. Thus, in the intima there is retention, modification and both intra- and extracellular accumulation of LDL particles and of the lipids they contain (Williams and Tabas 1995). Steinberg and coworkers have formulated the hypothesis that oxidative modification increases the atherogenicity of LDL (Steinberg et al. 1989). This theory is based on the concept that the oxidative modification, which is likely to occur mainly in the artery wall, promotes the uptake and retention of circulating LDL in the vessel wall. It is now well established that oxidative modification of LDL is important and possibly obligatory in the pathogenesis of the atherosclerotic lesion (Witztum 1994). Oxidative modification of LDL makes it susceptible to uptake by the scavenger receptor on macrophages (Steinberg et al. 1989) or by CD36 (Endemann et al. 1993), leading to formation of foam cells. Furthermore, many macrophages in the atherosclerotic lesions of cholesterol-fed rabbits express Fc and C3b receptors (Fowler et al. 1979) which also induce foam cell formation when exposed to ICs containing LDL (Fowler et al. 1979). Experimental studies show that LDL incubated with arterial endothelial cells or SMCs was converted to a form that was rapidly taken up by macrophages (Henriksen et al. 1981; Henriksen et al. 1982; Henriksen et al. 1983). This cell-mediated modification is indeed oxidative

modification of LDL particles (Morel et al. 1984; Steinbrecher et al. 1984). Macrophages are also capable of oxidative modification of LDL (Steinberg 1997b). Oxidation of LDL can be done in vitro by incubation with metal ions (e.g. copper or iron) and also by malondialdehyde (MDA).

There is now substantial evidence that oxLDL is present in vivo within atherosclerotic but not normal blood vessels.

- LDL isolated from human and animal atherosclerotic lesions, but not from normal arteries have all of the physical, chemical, immunological, and biological properties of oxLDL (Tsimikas and Witztum 2001)
- Atherosclerotic lesions contain immunoglobulins that recognize oxLDL (Ylä-Herttuala et al. 1994)
- Monoclonal antibodies to epitopes of oxLDL immunostain animal and human atherosclerotic lesions (Tsimikas et al. 1999)
- Radiolabeled oxidation-specific antibodies image oxLDL in the artery of live animals (Tsimikas, 1999; Tsimikas, 2000)

Oxidation of subendothelial LDL attracts monocytes, which enter the subendothelium and changes into macrophages, which in turn take up oxidized LDL and form foam cells.

It was recently shown that oxLDL levels in serum correlate with the severity of acute coronary syndromes. The more severe lesions also contain a significantly higher percentage of oxLDL-positive macrophages suggesting that increased levels of oxLDL are related to plaque instability (Ehara et al. 2001). Antioxidant drugs can slow the progression of atherosclerosis in experimental animal models (Kita et al. 1987; Steinberg 1997a; Tsimikas and Witztum 2000).

OxLDL is immunogenic in nature and has the ability to induce autoantibody formation (Esterbauer et al. 1992; Mironova et al. 1996; Palinski et al. 1990; Salonen et al. 1992). Concentrations of plasma autoantibodies to oxLDL correlate with both the accumulation and depletion of arterial oxLDL (Tsimikas et al. 1999; Tsimikas et al. 2000). Antibodies binding to MDA-LDL (an epitope on oxLDL) are predictive of the rate of atherosclerotic disease progression (Witztum and Palinski 1996). Several studies suggest that high levels of autoantibodies against oxLDL were predictive of atherosclerosis and its complications (Raitakari et al. 1997), MI (Wu et al. 1997), carotid atherosclerosis (Salonen et al. 1997). However, there are also studies showing no association between autoantibodies to oxLDL and the extent of atherosclerosis (Cherubini et al. 1997; Uusitupa et al. 1996; van de Vijver et al. 1996). Further, immunization of rabbits with oxLDL (Ameli et al. 1996) or with homologous MDA-LDL (Palinski et al. 1995) to raise the concentration of antibodies actually inhibits lesion progression. Thus, the reports on anti-oxLDL are inconsistent and hence their pathophysiological role in atherosclerosis is not yet fully understood.

Autoantibodies to cardiolipin

Antiphospholipid antibodies (aPL) constitute a group of antibodies that bind to anionic phospholipids such as phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), and di-phosphatidylglycerol (cardiolipin). Hughes and Harris described the antiphospholipid syndrome (APS), as a clinical syndrome characterized by aPL, venous and arterial thrombosis, recurrent abortion, and thrombocytopenia (Harris et al. 1987; Hughes 1985). The clinical features of APS may be seen in patients with no other concomitant disease process, the primary APS, or occur in association with SLE or

other autoimmune and inflammatory disorders, secondary APS (Hughes and Khamashta 1994). Two methods are currently used to detect aPL, the lupus anticoagulant (LA) test and the ELISA aCL test (Love and Santoro 1990).

aPL in patients with APS may be directed at least in part against plasma phospholipid-binding proteins, such as beta 2-glycoprotein I (β 2 GPI) or prothrombin. These proteins are involved in the control of thrombosis and haemostasis. It is postulated that bound β 2 GPI forms the antigen to which aPL antibodies are directed (McNeil et al. 1990).

β 2 GPI was found recently in the atherosclerotic plaques (George et al. 1999), and antibodies against it has been suggested to have a role in human atherosclerosis. These antibodies are associated with arterial and venous thrombosis in SLE and APS and can enhance the accumulation of oxLDL into macrophages (Hasunuma et al. 1997).

β 2 GPI is believed to play a role in the human coagulation system by inhibiting the intrinsic coagulation pathway and ADP-mediated platelet aggregation (Nimpf et al. 1987).

Prothrombin is an antigenic target of some aPLs and a subgroup of these antibodies possesses LA activity. LA is not directed to phospholipids alone, but presumably recognize an epitope that is exposed during binding of prothrombin to phospholipids (Beveris et al. 1991). Some IgG aPLs require prothrombin but not β 2 GPI as a cofactor. Antibodies to prothrombin were associated with MI in a prospective study of healthy dyslipidemic men (Vaarala et al. 1996). The mechanisms involved with the development of atherothrombosis may be related to the procoagulant activity of these antibodies in vivo. Due to their cross-reactivity with plasminogen, they may interfere with fibrinolysis. Some aPLs may also interfere with protein C and protein S-mediated inactivation of factor Va

and VIIIa, and may inhibit phospholipid-dependent activation of protein C by occupying the phospholipid surface (Keeling et al. 1993).

Cardiolipin, a phospholipid found in beef heart, was isolated in 1941 (Pangborn 1941). It is a constituent of the mitochondrial membrane and can be extracted from a number of natural sources such as bovine heart, liver and kidney (Fleischer 1967). Elevated aCL levels was observed in a highly selected series of young patients with MI (Hamsten et al. 1986). aCL have been described both in retrospective and prospective studies as a marker for atherosclerotic vascular disease (Adler et al. 1995; APASS 1993; Nityanand et al. 1995; Vaarala et al. 1995; Wu et al. 1997). Recent studies suggest that autoantibodies binding to cardiolipin, modified LDL and prothrombin can be used as markers of atherosclerosis (Vaarala 2000b; Vaarala et al. 1996). Immunization of LDL-receptor knockout mice with human aCL from an APS patient enhanced atherogenesis (George et al. 1997).

An antigen that triggers the formation of aCL can be of exogenous and/or endogenous origin. Many microorganisms have phospholipids on their surface and are known to induce an immune response against cardiolipin (Mattila et al. 1989; Vayssairat et al. 1995). Endothelial cell injury may lead to the exposure of antigens that are normally secluded within the phospholipid bilayer of the cell membrane, thus eliciting an aCL response (Cheng 1994).

The precise mechanism of action of aCL is not clearly understood. aCL coupled with antigen tends to form ICs and can activate the complement cascade. The terminal components of the complement cascade (MAC), C5b-9 was increased in patients with cerebral ischemia and aCL, a compelling evidence of complement activation by aCL (Davis and Brey 1992). aCL have been shown to bind to endothelial cells (Brey and Coull 1992; Hess et al. 1993) and can also bind to the phospholipid epitopes on

platelet membranes causing platelet activation and aggregation (Årfors et al. 1996).

AIMS OF THE PRESENT STUDY

To clarify the importance of immune mechanisms in atherosclerosis and its clinical complications, we investigated retrospectively and prospectively the role of autoantibodies, CICs and complement C4 in atherosclerotic vascular disease.

The objective of the present study was to investigate

- The association of CICs to atherosclerosis
- The association of autoantibodies against cardiolipin and oxidatively modified LDL to atherosclerosis
- The pathological immune response against alimentary proteins in patients after MI
- The association of complement factor C 4 allotypes to CICs and atherosclerosis

STUDY POPULATION

Paper I. Samples were collected from 2322 males at 50 years of age in Uppsala during 1970 to 1973. This cohort made up 82% of all 50 year old men living in Uppsala at that time. Of 257 men enrolled in this study, 119 men (mean age 49.6 years) developed MI between 50-70 years of age. A control group of 138 individuals was selected by simple random selection (mean age 49.6 years) from those who did not develop MI up to 70 years of age.

Paper II. Samples were collected from 74 patients out of 92 dyslipoproteinemic men with a first MI before the age of 45 years who were enrolled in the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT). Following a baseline coronary angiography they were randomly assigned to treatment for 5 years with bezafibrate or placebo (39 treated with bezafibrate, 35 with placebo) and underwent two angiograms at 2 and 5 years. Samples were obtained at baseline and at 2 and 5 years.

Paper III. A group of 81 consecutive patients (mean age 39 years, 72 men and 9 women) who had survived a first MI before the age of 45 years were enrolled in the study. Two control groups were examined together with the patients. One group consisted of age-matched, randomly selected, population-based healthy individuals, 79 men and 11 women. An additional control group used only for the determination of serum antibodies against some of the alimentary proteins consisted of 139 healthy blood donors, 95 men and 44 women, with a mean age of 42 years.

Paper IV. Sixty-two patients (mean age 49.3 years, 33 males and 29 females) who had been operated for atherosclerotic peripheral vascular disease before 50 years of age were considered eligible for the study. Sixty-seven (mean age 49.3 years, 34 males and 33 females), age and sex matched controls were randomly selected from the population based register of the Stockholm County. A pre-operative angiogram had been performed in every patient. The diagnosis of atherosclerotic vascular disease was made on the basis of clinical and angiographic features.

Paper V. Sera of 38 IDDM patients, 20 male and 18 female (mean age 46.8 years) were studied. The patients had been selected from a group of patients who were observed for 7 years. Half of the patients had developed vascular complications at time of study. The groups were matched with regard to age, sex and duration of disease. The control group was composed of 102 (mean age 47.8 years) age and sex matched healthy individuals. Of the 102 healthy controls, 56 were males and 46 were females.

METHODOLOGY

Isolation and quantitative determination of CICs

Complexes in the circulation are found bound to erythrocytes and free in plasma. To obtain more accurate assays of free complexes, the erythrocytes should be rapidly separated from the plasma to prevent the release of bound complexes.

There are many techniques for detecting and quantifying ICs.

They could be divided into two main groups: physicochemical and biological methods, based on the specific properties of complexed immunoglobulin molecules as compared to free immunoglobulin molecules (Table 3).

Table 3. Some of the commonly used antigen non-specific methods for detection of immune complexes

Analysis based on:	
Physical properties	Biological properties
<ul style="list-style-type: none"> • Analytical Ultracentrifugation • Gel filtration • Cryoprecipitation • Precipitation in polyethylene glycol (PEG) 	<ul style="list-style-type: none"> • Interaction with complement e.g. solid-phase C1q-binding tests • Interaction with conglutinin e.g. solid-phase conglutinin-binding tests • Interaction with complement receptors on cells e.g. Raji cell assay

The sensitivity of these techniques is generally good, though it is important to avoid all procedures that can generate non-specific aggregates for example repeated thawing and freezing of samples. In order to obtain a quantitative separation of ICs, we have used analysis based on the physical properties of the ICs, such as sucrose density ultracentrifugation and gel filtration.

Sucrose gradient ultracentrifugation:

Advantage:

- Physical separation of the ICs.

Disadvantage:

- Low ionic strength, risk for aggregation of the ICs.

Gel filtration:

Advantage:

- Physical separation of the ICs.

Disadvantage:

- High ionic strength, possibility of dissociation of ICs.

Precipitation by polyethylene glycol (PEG):

Advantage:

- Rapid and physical separation of the ICs.
- By using low concentration (3.5-4%), precipitation of monomeric immunoglobulins decreased significantly, without preventing precipitation of ICs formed in vivo.

Disadvantage:

- Lacks specificity i.e. possibility of precipitating other elements such as lipoproteins.

These methods are preparative, allowing not only detection, but also further immunochemical and biological characterization of the ICs. They are specific and sensitive. The isolated ICs can be accurately quantitated and analyzed for their constituents.

Widely used routine tests are based on the biological properties of ICs, such as conglutinin binding (interaction with conglutinin), C1q binding (interaction with complement), and Raji cell assay (interaction with complement receptors on cells) (Brown et al. 1983; Devey et al. 1980).

Advantage:

- These assays are easy to perform.

Disadvantage

- These methods depend on the characteristics of the CICs. Thus, different results may be obtained using different methods.

Determination of alimentary antigens in ICs

SDS-gel electrophoresis was performed using the PhastGel system, under reducing and non-reducing conditions. Western blotting with immunogold silver staining was performed with the Silver Enhancement Kit according to the instructions of the manufacturer with some modifications. The molecular size markers were stained with PhastGel Blue R (Pharmacia). The primary antibodies used were monoclonal anti-BSA, anti-chicken egg albumin and anti-gliadin (Paper III).

Analysis of serum lipids and lipoproteins

The major serum lipoprotein-that is, VLDL, LDL and HDL-were determined by a combination of preparative ultracentrifugation and precipitation of apo B-containing lipoproteins, followed by lipid analyses (Carlson 1973). Cholesterol (Zlatkis et al. 1953) and triglycerides (Fletcher 1968) were quantified after chloroform-menthol extraction (Carlson 1963) of whole serum, the VLDL fraction, the infranatant after ultracentrifugation (containing LDL and HDL), and the supernatant (HDL) after precipitation.

Oxidation of LDL

In our studies we used both copper (Cu^{++}) oxidized (Wu and Lefvert 1995) and MDA modified LDL (Zhou et al. 1998) as antigens to detect antibodies against oxLDL. To confirm the extent of oxidation, prepared

oxLDL was run on agarose gel electrophoresis and also measured by spectrofluorometry at 400/470 nm.

A broad spectrum of immunogenic oxidative epitopes are formed in vivo, and many of these epitopes occur in atherosclerotic lesions as well as on circulating LDL (Palinski et al. 1996). When polyunsaturated fatty acids in phospholipids and cholesteryl esters in the lipoprotein core undergo peroxidation, highly reactive breakdown products are formed, such as MDA and 4-HNE. These may then form highly immunogenic adducts with lysine and histidine residues of apo B (Palinski and Witztum 2000). MDA-LDL is a prominent epitope of oxLDL and present in atherosclerotic lesions. LDL is a large molecule and different epitopes may be formed during oxidation. This is why we have used both MDA modified and conventional copper modified LDL as antigens in our assay.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a sensitive assay for detecting antibodies against food proteins, cardiolipin, oxLDL, LDL and MDA-LDL. We used Harris method for standardization of antibodies against cardiolipin with little modification as described in detail in Paper I and IV (Harris et al. 1987).

Advantage of the method:

- Sensitive and simple to perform
- Excellent reproducibility
- Does not require radioactive isotopes
- A large number of samples can be run at the same time
- Both antibody and antigen can be detected and quantified
- Relatively cheap method; high benefit-cost ratio

We have used both mean +2SD and values above 95% confidence interval of the control population as a cutoff level for calculating the

prevalence of the antibodies. Thus, skews of data are not hidden. Levels of antibodies were expressed as OD values. Samples from each patient and the corresponding controls were run in the same plate at the same time.

C4 allotyping

C4 allotyping was done by flat bed agarose gel electrophoresis, according to the method of Zhang *et al.* and Awdeh *et al.* with some modifications (Awdeh and Alper 1980; Zhang *et al.* 1988). The two isotypes of C4: C4A and C4B have different electrophoretic mobility. Based on this property, C4 allotyping was done by agarose gel electrophoresis, followed by immunofixation of the separated bands by rabbit anti-human C4 antibody. Standard samples for the C4 allotypes frequently seen in the Caucasian population (A2, A3, A6, B1, B2 and B3) were included in each gel. C4 allotypes were assigned using published criteria (Mauff *et al.* 1983). When bands were intermediate between the A and B loci, C4B gene products were distinguished by their greater haemolytic capacity (Awdeh, Alper 1980). 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 (Kramer *et al.* 1989; Zhang *et al.* 1988). This method can not detect samples with one null alleles at both the A and the B loci, and the number of the null alleles may thus be underestimated. There is a quantitative variation in the relative expression of C4 genes in extended and non-extended MHC haplotypes, and the relative amounts of C4 protein variants in serum may not be regulated only by the number of expressed structural C4 genes, but also by other mechanisms (Truedsson *et al.* 1989). This makes diagnosis of heterozygous deficiency even less

accurate. There are also monoclonal antibodies available for determination of the concentrations of C4A and C4B. We have used both methods in our laboratory and have found a good correlation between electrophoresis and ELISA methods. However, ELISA is not accurate for determining heterozygous C4A or C4B deficiency.

RESULTS AND DISCUSSION

CICs as predictor for MI and coronary atherosclerosis

A major impediment in understanding the pathogenetic significance of these CICs in MI patients has been that previously published results were inconsistent and most of these studies have been retrospective. After MI, elevated CICs levels could be related to the event or secondary to e.g. myocardial necrosis, and hence have a very different etiology than CICs before such an event.

For such reasons, we have investigated humoral immune factors, including CICs, in atherosclerotic vascular disease using both retrospective and prospective study designs. A prospective nested case-control study, in which healthy 50-year-old men were followed for 20 years, showed that elevated levels of CICs are present before the MI, and thus might be related to the development of MI (Paper I). Both the prevalence and concentration of CICs were higher in men who developed MI than in those who remained healthy (Table 4).

Table 4. Prevalence and concentration of CICs in men who developed MI compared to those who remained healthy

Study design	CICs	MI (n=43/119)	Healthy (n=18/138)	<i>P</i>
Prospective (20 years follow-up)	Prevalence %	36	13	< 0.0001
	Concentration (mg/L)	135	70	< 0.0001

CICs, circulating immune complexes; MI, myocardial infarction; n, number of men with CICs.

The exciting finding in this study was that the concentration of CICs at 50 years of age was associated with an increased risk for future MI. The uncorrected odds ratio was 3.17 ($P < 0.0001$), whereas the odds ratio corrected for the conventional risk factors was 2.95 ($P < 0.0001$). The

concentration of CICs at age 50 years was related not only to future MI but also to death by MI (Table 3, Paper I).

CICs was proven in our study to be an independent and impetus risk factor for the development of atherosclerosis and MI.

In the second prospective angiographic study of dyslipidemic men with premature coronary artery disease, the serum levels of CICs were associated with progression of coronary atherosclerosis (Paper II). CICs increased dramatically over time in all patients, and this was independent of the lipid-lowering treatment with bezafibrate (Table 5).

Table 5. Effects of treatment and time on the concentration of CICs in patients after myocardial infarction

Study design	Time	CICs concentration (mg/L)		<i>P</i>
		Treated (n =39)	Placebo (n =35)	
Prospective (5 years follow-up)	Baseline	27±29	64±31	} < 0.0001
	Two years	289±29	287±31	
	Five years	230±29	170±31	

CICs, circulating immune complexes; n, number.

However, patients with relatively lower values of CICs had more progression of the atherosclerotic lesions. The molecular size of the CICs observed by us was in the range that is pathogenic (300-900 kDa) and likely to get deposited in vessel walls. Our finding was in agreement with earlier results showing that persistent high concentrations of CICs is important for vascular damage (Haakenstad et al. 1982; Lefvert et al. 1995). One reason for the apparently paradoxical finding of the relatively lower concentrations of CICs in more advanced disease, might be deposition of ICs in atherosclerotic lesions.

CICs after MI, in peripheral atherosclerosis and IDDM

We found that the prevalence and concentration of CICs were higher in patients after MI, in peripheral atherosclerosis and IDDM in comparison with the healthy controls (Table 6).

Table 6. Prevalence (%) and concentration of CICs (mg/L) in subjects with precocious MI, peripheral atherosclerosis and IDDM compared to controls

Study	Design	CICs	Patients	Controls
Precocious MI (Paper III)	Retrospective (Patients=76) (Controls=229)	Prevalence	18	5
		Concentration	136	45
Peripheral atherosclerosis (Paper IV)	Retrospective (Patients=62) (Controls=67)	Prevalence	50	10
		Concentration	90	35
IDDM (Paper V)	Retrospective (Patients=38) (Controls=102)	Prevalence	83	5
		Concentration	67	31

CICs, circulating immune complexes; MI, myocardial infarction.

Furthermore, both the prevalence and level of CICs were higher in IDDM patients with vascular complications (microangiopathy, nephropathy, peripheral polyneuropathy and foot ulcers) in comparison with IDDM patients without complications (Table 7).

Table 7. Prevalence and concentration of CICs in IDDM patients with and without signs of vascular complications

CICs	Patients with complications	Patients without complications	<i>P</i>
Prevalence %	91	68	< 0.05
Concentration (mg/L)	67	38	< 0.05

CICs, circulating immune complexes; IDDM, Insulin dependent diabetes mellitus.

Persistently high concentration of CICs can result in perturbation of endothelial cells and activation of the complement cascade. The net effect is a low-grade chronic inflammation of the vascular wall, with infiltration of monocytes and lymphocytes into arterial intima. Such an

inflammation resembles the basic features of atherosclerosis (Hansson et al. 1989).

An inflammatory reaction in the atherosclerotic vessel wall results in an increased oxidative capacity of various cell types (Glass, Witztum 2001; Libby 2001; Vaarala 2000a) causing peroxidation of lipids. These oxidatively modified LDL molecules can induce chemokines and expression of endothelial adhesion molecules in vitro (Klouche et al. 1999; Shih et al. 1999), resulting in infiltration of inflammatory cells. T-cell clones reactive with oxLDL derived from human atherosclerotic plaques secrete high levels IFN- γ , indicative of a Th1-type reactivity which is associated with inflammation (Stemme et al. 1995). Thus, the elevated levels of CICs found by us can have an important pathogenic role for the initiation and/or enhancement of atherosclerotic processes. Furthermore, CICs may induce platelet activation and aggregation and hence enhance thrombus formation (Clark et al. 1982).

Contents of CICs

What induces the formation of CICs and what predisposes to persistence of CICs in these subjects is still not known. One or several factors could be implicated. Persistent antigenic stimulus (endogenous or exogenous) could be one factor. Impaired clearance of CICs could be another important factor. Chronic microbial infections, abnormal reactions against alimentary antigens and endogenous antigens like oxLDL or cardiolipin could cause such persistent immune stimulation.

We investigated the constituents of the CICs in patients after MI who had persistently high concentrations of CICs five years after the event (18% of all patients). Alimentary antigens/antibodies in immune complex form were present in seven patients out of 14 (50%) (Paper III). The 14

patients with CICs had lower plasma fibrinogen levels (median value 3.05) than patients without CICs (median value 3.5 g/L, $P < 0.01$). Patients with alimentary antigens/antibodies in CICs had lower serum triglycerides ($P < 0.05$) and VLDL cholesterol values ($P < 0.05$) than the other patients with CICs. Our findings indicate that these patients constitute a special group less impeded with conventional risk factors. In these patients, an aberrant immunological reaction may be a primary factor responsible for the development of MI.

When we determined the antibody isotypes in these CICs, IgG was the predominant antibody species, followed by IgM whereas the IgA content was low. In one study, IgA2-ICs provided the strongest stimulus for neutrophil activation in vitro (Zhang et al. 1995). However, IgA-CICs do not readily activate the complement system or promote phagocytosis (Heremans 1974). IgA-CICs thus represent the normal and harmless way of eliminating food proteins, whereas the IgG-CICs present in all our 14 patients should be considered as potent initiators of vascular damage. The role of CICs in the activation of the complement cascade was clearly seen when four young MI patients who had alimentary antigens/antibodies in CICs were subjected to a 2-week elimination diet followed by challenge with cow milk.

A rise in CICs, activation of the complement system and high plasma concentration of vWFAg was evident within one week (Table 8). The MAC complex displaces membrane phospholipids, leading to formation of a transmembrane channel that enables the passage of ions and small molecules, which results in damage to the cells (Kuby 1994). The elevated levels of vWFAg found in all our patients after the challenge suggests that CICs caused a significant perturbation of endothelial cells, which are the main source for vWFAg.

Table 8. Maximal increases of CICs, complement factors 3a, 3d, C5b-9 and vWFAg after milk provocation in four patients with previous myocardial infarction at a young age. These values had reverted to baseline level by the end of the second week.

Patient	Maximal increase (%)				
	CICs	C3a	C3d	C5b-9	vWFAg
1	345	425	300	817	800
2	2067	216	175	188	425
3	213	438	162	158	960
4	242	177	118	120	328

CICs, circulating immune complexes; C3a, complement factor 3a; C3d, complement factor 3d; C5b-9, terminal complement complex (MAC); vWFAg, von Willebrand factor antigen.

Elevated levels of vWFAg was an independent risk factor for the acute coronary syndromes (Thompson et al. 1995) and independently predicted recurrent MI and death by MI (Jansson et al. 1991). vWFAg has an important role in platelet adhesion to subendothelial structures (Sadler 1991). Furthermore, an increase in vWFAg concentration may also increase the risk of thrombus formation.

We also investigated the constituents of the CICs in six patients with IDDM and found relatively higher concentrations of aCL in these CICs as compared to serum (Table 1, Fig. 3a-c. Paper V). Particularly, CICs-derived IgA-aCL was 42-163 times higher than serum-derived IgA-aCL, (for IgG-aCL 2-8 and IgM-aCL 3-25 times). Such a concentration of aCL in complex form was also present in our earlier study (Årfors and Lefvert 1997). The presence of aCL reactivity in complexes might bestow them with special pathogenic potentials, for example with regard to binding to membrane phospholipids.

Complement C4

The role of C4 for the tendency to immune complex formation was investigated in patients with MI, peripheral atherosclerotic and IDDM, and the following findings emerged:

- Healthy men homozygous for C4Q0 who developed MI between the age of 50-70 years had higher levels of CICs than men who remained healthy ($P < 0.01$). The prevalence of CICs was higher in men who developed MI and were homozygous for C4Q0 (86%) than those developed MI and were heterozygous for C4Q0 (36%) ($P < 0.03$) (Paper I).
- All postinfarction patients with CICs had partial complement C4 deficiencies (Paper III).
- Patients with peripheral atherosclerosis with C4Q0 had significantly higher prevalence of CICs than patients without C4Q0 (65% and 38%, respectively). All patients with homozygous C4A*Q0 and 82% with heterozygous C4A*Q0 had CICs (Paper IV).
- The prevalence of CICs was higher in IDDM patients with C4A*Q0 than in patients with no C4A*Q0 (96% *versus* 69%, $P < 0.05$). Patients with vascular complications had CICs in 91% and those without complications in 68% of cases ($P < 0.05$). Patients with vascular complications had higher prevalence of C4A*Q0 than of C4B*Q0 (49% *versus* 15%, $P < 0.01$) (Paper V).

Our results strongly support the role of C4A deficiency for immune complex formation.

The higher prevalence of C4A*Q0 in IDDM patients with vascular complications, further supports the role of C4A and suggests that patients

with C4A*Q0 are specially predisposed to immune complex induced vascular complications.

Thus, chronic persistent CICs associated with genetic deficiencies of C4 might be an additional etiological factor for the development of chronic vascular damage resulting in premature atherosclerosis.

Autoantibodies as a predictor for MI and coronary atherosclerosis

In our studies, we have measured aCL both in prospective (MI) and retrospective (IDDM) study designs. Elevated aCL levels were observed in young postinfarction patients (Hamsten et al. 1986) and high levels aCL have been described as an independent risk factors for MI or cardiac death in middle-aged men (Vaarala et al. 1995; Wu et al. 1997). aCL have been described as an independent risk factor for atherosclerotic vascular disease. It has been suggested that aCL should be routinely measured in all patients at risk for atherosclerotic vascular disease, and in those in whom thrombosis is a major pathoetiology (Glueck et al. 1999). We found that the level of IgG-aCL and the prevalence of both IgG- and IgM-aCL were higher in subjects who developed MI and had CICs compared to subjects without CICs (Paper 1). There was also a positive correlation between the levels of CICs and IgG-aCL in men who developed MI ($r=0.4$, $P= 0.0001$). This suggests, that aCL could be involved in the formation of CICs in these patients.

Patients with MI followed for five years showed a decline in the levels of aCL with time (Paper II). This might be the result of elimination, perhaps through binding to exposed phospholipids on the damaged arterial wall or by formation of CICs. Antibodies to cardiolipin seem to have an increased tendency to immune complex formation. Our present study

supports our earlier data showing that these antibodies might be involved in the formation of CICs (Årfors, Lefvert 1997).

Extensive data have accumulated on the role of autoantibodies against epitopes of oxLDL in humans, suggesting that the level of such autoantibodies may be an indicator of the severity of atherosclerosis and its rate of progression. Salonen *et al.* observed, that elevated levels of autoantibodies to oxLDL was an independent predictor of lesion progression in carotid atherosclerosis (Salonen et al. 1997).

Subsequently, many studies have reported elevated autoantibody levels to oxLDL in subjects with increased frequency of risk factors or clinically manifest atherosclerosis, including coronary artery disease, MI, peripheral vascular disease, hypertension and pre-eclampsia (Bergmark et al. 1995; Branch et al. 1994; Maggi et al. 1993; Puurunen et al. 1994; Virella et al. 1993; Wu et al. 1997).

In our study, we found that elevated levels of IgM and IgG antibodies to oxLDL were associated with a more rapid progression of atherosclerotic lesions and the occurrence of new occlusions (Paper II). This relation was independent of established risk factors. Ehara *et al.* observed that levels of oxLDL were related directly to the severity of coronary syndromes (Ehara et al. 2001). High concentrations of autoantibodies to oxLDL observed by us reflect an ongoing antigenic challenge produced by the continuous generation of oxLDL. The generation of both IgM and IgG antibodies suggests, that both T cell-dependent and -independent pathways for B cell activation are involved in the immune response against oxLDL.

Autoantibodies to oxLDL were also found to be present as part of immune complexes with oxLDL (Lopes-Virella et al. 1999; Wu, Lefvert 1995), and can recognise material in atherosclerotic lesions of rabbits and

man (Haberland et al. 1988; Ylä-Herttuala et al. 1994). High concentration of CICs containing oxLDL has been suggested as a risk factor for the development of CAD in IDDM (Lopes-Virella et al. 1999). Furthermore, it has been reported that ICs derived from SLE sera stimulate the accumulation of cholesterol in cultured SMC from human aorta (Kabakov et al. 1992). Thus, one pathogenic effect of the autoantibodies to oxLDL might be participation in the formation of ICs. The data obtained by us extend the findings of several previous studies and support that high concentrations of autoantibodies against oxLDL are related to the development of atherosclerosis.

Autoantibodies in IDDM

The prevalence of autoantibodies to cardiolipin in IDDM patients with and without signs of vascular complications and in control is shown in Table 9.

Table 9. Prevalence (%) of aCL in IDDM patients with and without signs of vascular complications and in controls

Ig type	All patients	Patients with complications	Patients without complications	Controls
IgG*	21	32	11	5
IgM	none	none	none	4
IgA**	45	47	42	7

Statistical comparisons were done between all patients and all controls.

aCL, anticardiolipin antibody; IDDM, Insulin dependent diabetes mellitus, Ig, immunoglobulin; *, $P < 0.005$; **, $P < 0.001$.

The levels of IgG- and IgA-aCL were higher in both patient groups than in the control group ($P < 0.001$). There was no difference in the levels of these antibodies between patient groups. The levels of IgM-aCL were higher in IDDM patients with vascular complications than in patients without or controls ($P < 0.005$).

One interesting aspect of our results is the higher concentrations of IgM-aCL in IDDM patients with vascular complications than in patients without or controls ($P < 0.005$). When pentameric IgM is bound to an antigen, at least three binding sites for C1q are exposed. Thus, due to its high avidity, IgM has a high capacity to activate the complement system. Another interesting aspect of our results is the elevated level of IgA-aCL in IDDM patients. The production of IgA-aCL can be triggered by intestinal or respiratory tract infections. Since patients with IDDM have an increased susceptibility to infections, IgA-aCL might represent cross-reacting antibodies that have been triggered by microorganisms bearing surface phospholipids. There was no increased prevalence or levels of antibodies against oxLDL in both patient groups when compared to controls.

CONCLUSIONS AND PERSPECTIVES

Taken together, the present studies revealed links between humoral immune factors and atherosclerotic vascular disease. We draw the following conclusions:

- CICs alone and in combination with aCL predict MI.
- CICs and autoantibodies against oxLDL are associated with progression of coronary atherosclerosis.
- Dietary proteins can induce formation of CICs and give rise to signs of vascular damage.
- CICs and aCL are associated to vascular complications in IDDM.
- C4Q0 enhances the propensity to immune complex formation.

The present work suggests that immune reactions are likely the main impetus in triggering and/or enhancing the atherosclerotic processes in a subgroup of individuals.

Patients with CICs and antibodies to cardiolipin and oxLDL should be followed with repeated determinations of these parameters, to further evaluate their role as predictors of atherosclerosis and its complications. In patients with persisting CICs, it is important to define the antigen(s) triggering formation of CICs. Elimination of the culprit antigen(s), e.g. food protein(s) from the diet or microorganism by anti-microbial therapy can result in disappearance of CICs from the circulation and thus decrease the risk for vascular inflammation. The mechanisms of action of these humoral immune factors need to be further studied.

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