Role of IgM and C-reactive protein in ischemia reperfusion injury

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Abbreviations

Abs	antibodies
AMI	acute myocardial infarction
Anti-Pc IgM	IgM antibody to phosphorylcholine
C1-inh	C1-Inhibitor
CK	creatine kinase
CPS	C-polysaccharide of pneumococci
CRP	C-reactive protein
CRs	complement receptors
ELISA	enzyme-linked immunosorbent assay
FcγR	Fc gamma receptor
HSA	human serum albumin
IRI	ischemia reperfusion injury
II/R	intestinal ischemia reperfusion
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
Lyso-Pc	lysophosphatidylcholine
mAb	monoclonal antibody
MAC	membrane attack complex
MBL	mannose-binding lectin
MFI	mean fluorescence intensity
NHP	normal human plasma
NRP	normal rat plasma
oxLDL	oxidized LDL
PAF	platelet activating factor
Pc	phosphorylcholine
PGE-2	prostaglandin E2
$sPLA_2$	secretory phospholipase A_2
SRs	scavenger receptors
TGF β	transforming growth factor-β
TNF α	tumor necrosis factor- α

CHAPTER 1

General introduction

Introduction

For decades, it is known that the innate immune system provides protection against invading pathogens. There is now much evidence that this system participates also in the recognition of self-antigens in eukaryotic organisms. Two proteins of the innate immune system, C-reactive protein (CRP) and natural anti-phosphorylcholine IgM antibody (anti-Pc IgM), share the ability to bind specifically to phosphorylcholine (Pc). Pc is exposed on many pathogenic microorganisms, including bacteria, fungi and parasites. In mammals, Pc is normally only present on cell membranes after cell damage; furthermore it may be present in lipoproteins after oxidation. Indeed, CRP and anti-Pc IgM bind to Pc in the membranes of damaged cells and to oxidized lipoproteins, but not to either viable cells or native lipoproteins. In addition to their shared ability to bind to Pc, CRP and anti-Pc IgM can activate the complement system via the classical pathway upon ligand recognition.

CRP is the prototypical acute-phase protein in humans. CRP was discovered in 1930 when it was shown that it could bind to C-*polysaccharide of pneumococci* [1]. It is now known that CRP specifically recognizes Pc [2]. However, CRP is not the only protein in plasma that binds to Pc. IgM antibody presents in sera of humans or animals binds also to Pc (anti-Pc IgM) [3-5]. Remarkably, in addition to their shared ability to bind to Pc, both proteins can activate complement upon ligand recognition [6-8]. Furthermore, CRP has been claimed to interact with Fc gamma receptors (FcγR), particularly FcγR II, although this interaction is weak and probably of limited biological significance [9-11]. IgM does not interact with FcγR, preferring instead to bind to the poly-Ig R and to Fc α/μ receptor [12,13]. There is accumulating evidence that complement activation by CRP and natural IgM antibody (IgM) is an important contributor to ischemia/reperfusion injury (IRI). In mouse models of IRI, a role of IgM herein has been postulated. In humans, both IgM and CRP play a role in complement activation after acute myocardial infarction (AMI). In rats, CRP-mediated complement activation seems to be involved in the increase of infarct size in rats subjected to coronary artery ligation, although it has been claimed that rat CRP (rCRP) does not activate rat complement. The relative contribution of both proteins in the development of IRI in humans and animals is still not fully elucidated. This chapter provides information about structural and functional aspects of CRP and anti-Pc IgM and their possible role in innate immunity, in atherosclerosis and in IRI. Below is summarized why nature has endowed us with two structurally different but functionally similar proteins.

Structure of CRP and anti-Pc IgM

CRP is a member of the pentraxin family and is characterized by a structure consisting of five non-covalently bound identical subunits (about 23 kDa). These subunits are arranged symmetrically around a central pore in an annular configuration. Each CRP subunit has a recognition face with a Pc binding site (consisting of two coordinated calcium ions) and an effector face with a C1q-binding site. The crystal structure of Pc bound to CRP has revealed that calcium ions and the residues Glu^{81} and Phe^{66} are important in ligand binding. Major protein-ligand interaction occurs between the protein-bound calcium ions and the phosphate groups of Pc and with Glu^{81} via the choline moiety [14]. The structure and topology of the C1q-binding site has been defined by site-directed mutagenesis. This site is located at the shallow end of the cleft of the effector face, and the residues Asp^{112} and Tyr^{175} are critical for the CRP binding to C1q [15].

Anti-Pc IgM belongs to a group of plasma proteins, the immunoglobulin family [16]. Immunoglobulin molecules have a basic structure consisting of two identical light (smaller) and two identical heavy (larger) polypeptide chains linked together by disulphide chains. The nature of the antigen-binding sites of anti-Pc antibodies has been studied. The sequence Phe-Tyr-Met-Glu found in the first complementary-determining region of the immunoglobulin heavy chain in 89 % of all anti-Pc Abs is involved in ligand-target interaction [17]. As in CRP, the residues Phe^{32} and Glu^{35} are involved in this interaction.

The fact that the CRP-Pc interaction is calcium-dependent whereas anti-Pc IgM binds ligands independently of calcium (unpublished observation Diaz Padilla N.) suggests that these proteins use different mechanisms to interact with Pc, regardless of the fact that the same two amino-acid residues are present in both Pc-binding sites. IgM circulates in plasma usually as a pentamer consisting of five basic units, and it is one of the largest plasma proteins (970 kDa) [16]. Because of its pentameric structure, IgM has 10 antigen-binding sites per molecule. This is in contrast to CRP, which binds only five Pc groups.

Phosphorylcholine: common ligand of CRP and anti-Pc IgM

Pc is the name for the phosphorylated choline head group found in phospholipids of many prokaryotes, i.e. bacteria, and is almost universally expressed in eukaryotes. Pc contains both positive and negative charges and is overall, electrically neutral (zwitterionic) over a wide pH range [18]. In prokaryotes, Pc is bound both to carbohydrate and lipid components (figure 1).

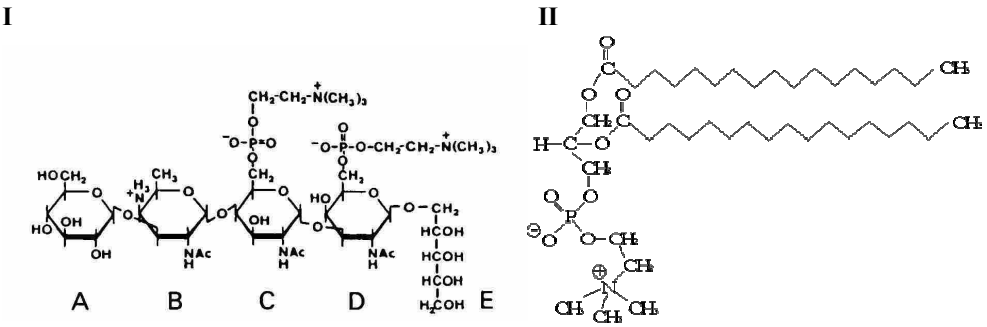

Figure 1. Structure of Pc bound to carbohydrates and lipids component. I) *Two Pc molecules are bound to CPS (teichoic acid) from streptococcus pneumoniae. In teichoic acid, the repeating units are linked by phosphodiester bonds between O5 of ribitol and O6 of the glucopyranosyl residue of adjacent units.* ***A****, D-Glcp;* ***B****, 2 acetamido-4 amino-2,4,6-trideoxy-D-galactose;* ***C****, D-GalpNAc;* ***D****, D-GalpNAc; E, D-ribitol (Fischer W.et al., 1993).* ***II)*** *Pc is bound to fatty acids to form phosphatidylcholine found in the cell membranes of prokaryotes.*

Indeed, it has been shown that CRP and anti-Pc IgM bind to Pc present in the *C-polysaccharide* from the cell wall of *S. pneumoniae* [1,19]. Interaction of CRP with Pc in lipopolysaccharides from *Haemophilus influenzae, Pseudomona aeruginosa, Neisseria meningitides, Neiserria gonorrhoeae and Proteus morganii* has been also reported [20-22]. In eukaryotes, Pc is the head group of the lipid phosphatidylcholine present in the outer leaflet of

the intact cell membranes and in lipoproteins such as low-density lipoprotein (LDL). However, Pc under normal conditions remains inaccessible, thereby preventing the interaction of CRP or anti-Pc IgM with viable cells and non-modified lipoproteins. Under certain conditions, Pc ligands are exposed, allowing their interaction with Pc-binding proteins (Table 1).

Table 1. Binding specificities of CRP and anti-Pc IgM

	CRP	**Anti-Pc IgM**
Pc on		
CPS *(S. pneumoniae)*	+++	+++
LPS (*H. influenzae)*	+++	+++
EAC	?	+++
LAC or NC	+++	+++
OxLDL	+++	+++
Oxidized Ptc	+++	+++
Staining of atheroma	+++	+++
AMI	+++	+++

Pc: phosphorylcholine; CPS: C-polysaccharide of pneumococci; LPS: lipopolysaccharide; EAC: early apoptotic cells; LAC: late apoptotic cells; NC: necrotic cells; OxLDL: oxidized low-density lipoprotein; Ptc: phosphatidylcholine; AMI: acute myocardial infarction.

Flip-flop and oxidation of cell membranes

Most cells are characterized by a marked asymmetry in the distribution of the phospholipids composing the plasma membrane. Most of the phosphatidylethanolamine (PE) and nearly all phosphatidylserine (PS) molecules are localized in the inner leaflet, and the majority of sphingomyelin and phosphatidylcholine (PC) are in the outer leaflet of the membrane. This asymmetry is maintained by an aminophospholipid translocase that sequesters PS and to lesser extent PE in the inner leaflet of the plasma membrane bilayer [23]. When cells undergo apoptosis, and upon depletion of ATP as seen during ischemia, this asymmetry is not maintained, resulting in exchange of phospholipids between the inner and outer leaflets with the exposition of PS and PE, the so-called flip-flop of the membrane [24]. At sites of tissue injury, polymorphonuclear leukocytes are recruited and activated among others by inflammatory cytokines [25]. Activated cells generate reactive oxygen species, leading to oxidation of the phospholipids, which may further disturb the tight package of lipid bilayer. Because of the loss of the membrane integrity, efficient hydrolysis of the phospholipids by cytosolic and secreted phospholipases A2 is facilitated, thus generating lysophospholipids of PC, PE and PS. Binding sites for CRP and anti-Pc IgM, lysophosphatidylcholine, are generating in the outer leaflet of the jeopardized membranes [26, 27], (figure 2).

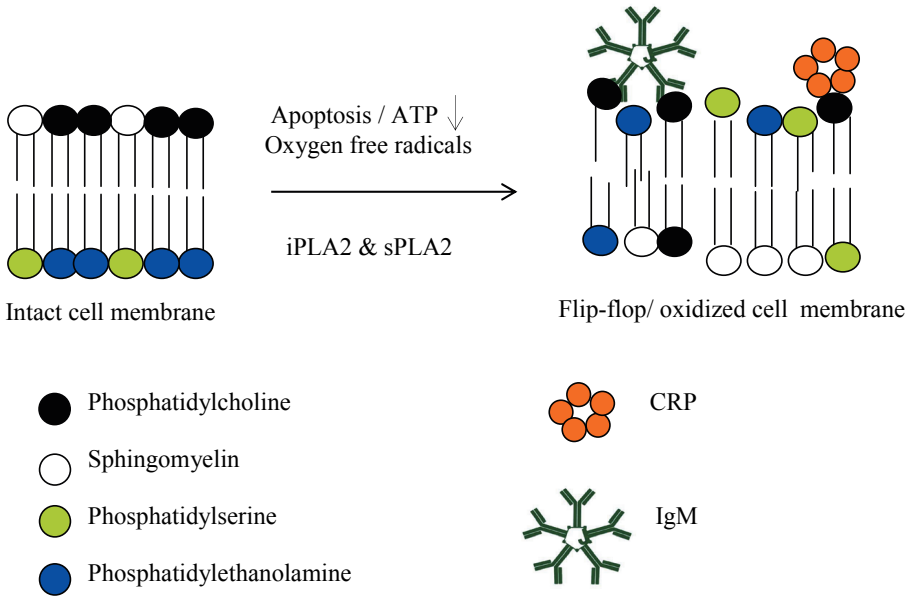

Figure 2. Proposed mechanism for CRP and anti Pc-IgM binding to Pc on damaged cell membranes. *In intact cells, sphingomyelin and phosphatidylcholine are present in the outer leaflet of the membrane. After apoptosis and/or upon depletion of ATP, the distribution of the phospholipids in the membrane is exchanged and PS and PE are present in the outer leaflet (flip-flop). Because of the flip-flop and oxidation of the phospholipids, the tight package of lipid bilayer is lost and the phospholipids are susceptible to hydrolysis by phospholipases A_2. In this way, lysophosphatidylcholine is generated in the outer leaflet of the membrane, thus creating binding sites for CRP and IgM.*

In vitro it has been demonstrated that CRP and anti-Pc IgM bind to Pc exposed in the membrane of apoptotic cells [8, 28,29]. In vivo evidence of IgM binding to Pc on damaged cells is supported by observation of decreased levels of these Abs in plasma from patients with skin tumors after treatment with tumor necrosis factor-alpha (TNF α). Massive death of the malignant and endothelial cells is observed following treatment [30,31]. CRP and IgM are also deposited in heart specimens from patients who have died from acute myocardial infarction**.** The deposition pattern of IgM in the tissue is similar to that of CRP, suggesting that both proteins recognize similar epitopes (Pc) in the ischemic heart [32]. Indeed, a considerable amount of lyso-phospholipids is generated in the infarcted myocardium [33].

Oxidized phospholipids on lipoproteins

Both proteins are also able to bind to Pc on LDL, but only after oxidation. In human atherosclerotic lesions, which are known to contain high amounts of oxidized phospholipids, positive staining for CRP has been found [34,35]. In addition, anti-Pc IgM Abs are also deposited in atherosclerotic lesions of rabbits and mice [19,36,37]**.**

Functional properties of CRP and anti-Pc IgM

CRP is produced by hepatocytes in response to an infection and/or an inflammatory stimulus [38]. The regulation of its synthesis varies among species. In humans and rabbits, normal levels of CRP are very low (about 1 mg/L) and can increase up to 1000-fold during an acute-phase response [39,40]. In rats, CRP is a minor acute-phase protein, with normal levels of about 500 mg/L, increasing at most 2-fold during infection and/or inflammation [41]. CRP levels in mice reach a maximum expression of 2-3 mg/L in the acute phase [11,42], (table 2).

Table 2. Behaviour of CRP and anti-Pc IgM in different species

Species	CRP normal plasma (mg/L)	CRP acute-phase plasma (mg/L)	anti-Pc IgM normal plasma (mg/L)
Man	1	1000	1.5
Rat	500	1000	?
Rabbit	1	1000	?
Mouse	<0.02	2-3	0.05-5

IgM produced by different types of cells can be found in the circulation of normal mice, rats and humans. Natural IgM is produced mainly by the B1 subset of B-lymphocytes without antigen exposure, and antigen-induced IgM is produced by conventional B-2 cells in the presence of an antigen [43]. B1 cells, characterized by the CD5 marker, are already present in the peritoneal cavity during fetal and neonatal life and produce IgM without antigen stimulation [44]. However, foreign antigens can stimulate B1 cells after complement activation. Complement receptors (CRs) CR1 and CR2 on B1 cells bind to activated complement fragments attached to an antigen-transmembrane IgM antibody complex. It seems that co-ligation of CR2 with the B-cell receptor leads to activation of the cells and enhances antibody production [45], (figure 3).

Natural IgM antibodies are polyreactive, since they can bind to several unrelated antigens with low affinity, such as nucleic acids, carbohydrates and phospholipids [44]. Natural IgM antibodies to Pc are present in normal serum of human, rat and mice [3-5]. In humans and mice these antibodies are frequently of the T15 idiotype (id).T15 id is characterized by a canonical V_HDJ_H-$V_\kappa 22J_\kappa 5$ H+L Ig chain combination. This combination of H+L chains was initially identified in an IgA protein secreted by the plasmacytoma line TEPC-15 and defines the T15 idiotype [37,46].

The contribution of both CRP and anti-Pc IgM to Pc binding varies in different species in the normal situation and during acute-phase reaction (APR). In normal human and rabbit plasma with similar levels of CRP and anti-Pc IgM, IgM could be the predominant ligand to Pc binding (human anti-Pc IgM is about 1.5 µg/mL, Diaz Padilla N.; unpublished observation). We suggest that the increase in CRP of about 1000-fold during APR in both species makes CRP the dominant Pc-binding protein during inflammation, infection, and tissue damage. In mice with high IgM and low CRP levels, IgM will be the dominant protein, whereas in rats with high CRP levels and low IgM, CRP will be the dominant factor.

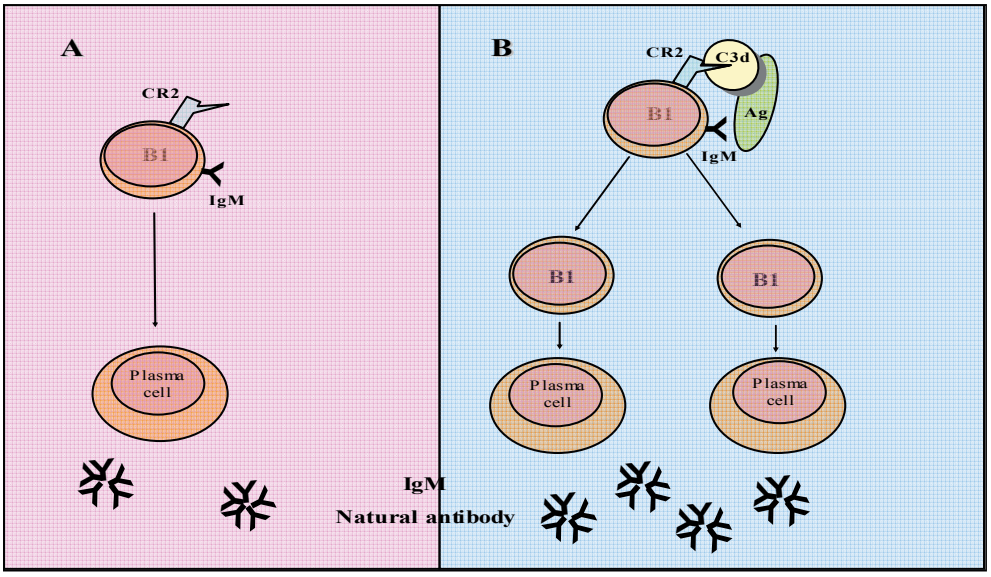

Figure 3. Schematic view of the role of the complement receptors in the production of natural IgM by B1 cells. A) *Natural IgM is produced by B1 cells in absence of antigen.* ***B)*** *Immune complexes formed by antigen (Ag) and transmembrane IgM (IgM) on the cell surface are suggested to lead to complement activation. Activated complement fragments such as C3d bind to complement receptors, i.e. CR2 on the cell membrane. The cross-linking of the complement receptor and B-cell antigen-receptor triggers proliferation of B1 cells leading to IgM natural antibody production.*

Complement activation by CRP and anti-Pc IgM

Principal biological consequences of CRP are largely determined by interaction with C1q upon Pc recognition, thereby activating the complement system [47]. Human, rabbit and rat CRP are able to activate complement via the classical pathway [7,48,49]. Activation of complement leads to either direct (via membrane attack complex, MAC) or indirect (via CRs on macrophages/dendritic cells), target destruction [50,51]. Complement activation triggered by CRP leads to deposition of activated complement components C4b, C3b, and C3bi on microbes, damaged cells, or OxLDL. Gershov *et al*. have demonstrated that CRP binding to apoptotic cells results in activation of early complement cascade events and in reduction of MAC assembly on apoptotic cells by recruitment of Factor H [52]. Factor H accelerates the decay of the C3 and C5 convertases. The decreased formation of MAC in membranes prevents leakage of intracellular constituents into the environment and in that way prevents increase of inflammation. Indeed, early complement components such as C4b, C3b/bi likely recruit and promote the removal of apoptotic cells by the CRs CR1, CR3 and CR4 on macrophages and dendritic cells. The uptake of complement-coated particles by CRs induces the production of non-inflammatory components such as TGFβ, PGE2, and PAF [53]. The uptake by macrophages and dendritic cells of necrotic cells, intracellular contents, OxLDL, and pathogens is enhanced through ligand binding to scavenger receptors. Macrophages

transformed to foam cells release proinflammatory cytokines, i.e. TNFα, IL-1, IL-12 (figure 4).

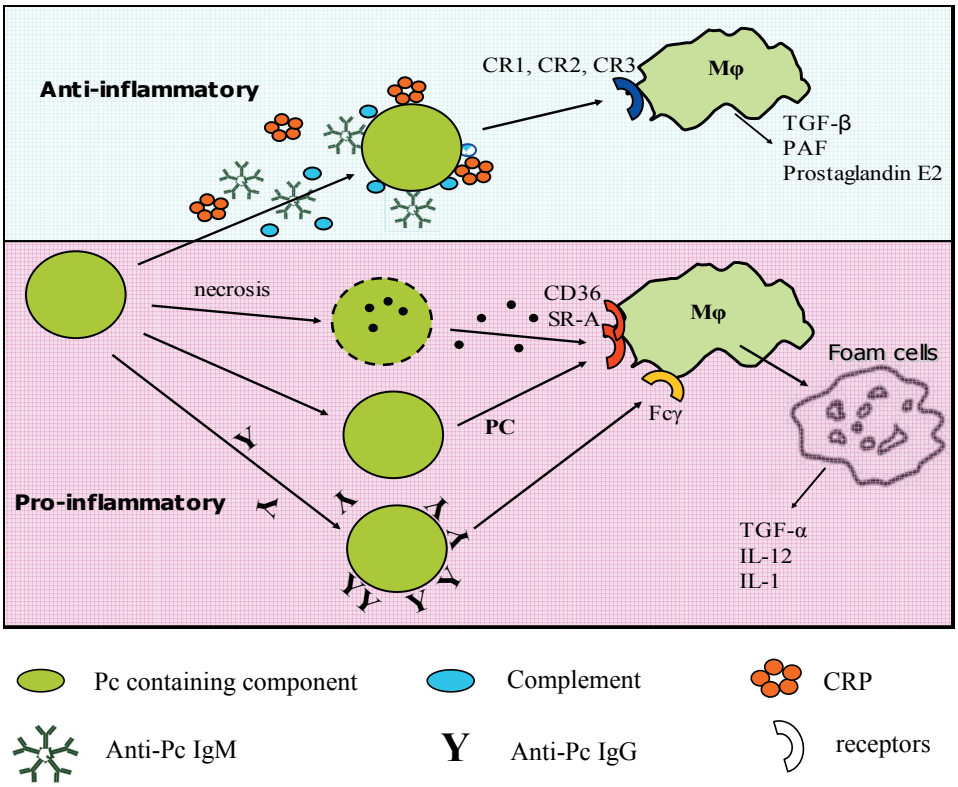

Figure 4. Proposed consequences of CRP-and anti-Pc IgM-mediated complement activation in the removal of Pc ligands. *Phosphorylcholine (Pc-containing component) is present in pathogens and in altered self-molecules (apoptotic or necrotic cells or OxLDL). In the normal situation, CRP and IgM bind Pc followed by complement activation. Complement components are deposited on pathogens and altered self- molecules. Complement receptors CR1, CR2, and CR3 on macrophages and dendritic cells bind complement-coated molecules. Immunosuppressive components, i.e. PAF, TGFβ and Prostaglandin E2 are secreted and promote the clearance in a non-inflammatory fashion. In situations of low CRP and IgM levels, altered self-molecules or pathogens are not efficiently phagocytosed. Apoptotic cells may undergo secondary necrosis and intracellular contents are released. The uptake by macrophages and dendritic cells of necrotic cells, intracellular contents, OxLDL, and pathogens is through the scavenger receptors (CD36 and SR-A). It induces the release of proinflammatory cytokines i.e. TNFα, IL-1, IL-12. In addition, IgG could recognize Pc on these components, which are taken up via FcγR on phagocytic cells. Phagocytosis via FcγR or scavenger receptors (SR) leads to an inflammatory reaction. In the presence of low amounts of complement-regulatory proteins, complement activation mediated by IgM may lead to pronounced MAC formation. MAC inserted in the cell membrane results in cell death and necrosis with release of intracellular contents.*

Anti-Pc IgM is also able to activate the classical pathway of the complement system after binding to Pc in microbes, and in altered self-molecules [8,29,54,55]. While one molecule of IgM can lyse a red blood cell, approximately a thousand IgG molecules are required to accomplish the same [56]. Because IgM is the most potent complement activator, it is expected that anti-Pc IgM-mediated activation may lead to increased MAC formation and to cell death. It has been shown that anti-Pc IgM-mediated activation is responsible for deposition of early complement components, i.e. C3b/bi, on the membrane of apoptotic cells [8]. However, substantial deposition of MAC after this activation has not been reported.

We suggest that this type of complement activation by both molecules, i.e. limited to generation of early components with reduced amounts of late complement components, may lead to efficient opsonization and removal, and is not a strong stimulus for inflammation.

CRP and anti-Pc IgM in the innate immune response

Nearly 20 years ago, it was demonstrated that human CRP mediates protection from *S. pneumoniae.* Overall survival of mice infected with *S. pneumoniae* increased when the mice were treated with human CRP [57,58]. The requirement of complement for the protective effect of CRP was demonstrated in mouse models. The protective effect of injected CRP to wild type, FcγR, FcγR IIb and FcγR III-deficient infected mice was abrogated by complement depletion with cobra venom factor. In addition, CRP failed to protect C3- or C4-deficient mice from pneumococcal infection [11]. It has also been suggested that CRP can bind to FcγR I and FcγR II on leukocytes [9,10]. This binding results in increased production of the early protective cytokines TNF-α and IL-1β in response to *S. pneumoniae* [59]. However, Saeland *et al*. have reported that CRP does not bind to FcγR IIa [60]. They claim that earlier findings were obtained because CRP-detecting IgG1 Abs may interact via the Fc portion with the FcγR. Saeland *et al*. used $F(ab')_2$ fragments of the mouse IgG1 anti-CRP Ab. The presence of impurities in insufficiently purified CRP preparations could explain previous findings of interactions between CRP and IgG receptors [61]. Finally, gamma-chain-deficient mice, which lack most effector functions of FcγR, are protected against pneumococcal infections after CRP injection, suggesting that the interaction of CRP with these receptors is not of essential importance for the antibacterial effects of CRP [11]. Thus, at present there is no convincing evidence for involvement of FcγR in the protective effect of CRP in microbial infections. It is therefore likely that the protective effector functions of CRP against bacterial infection result from activation of complement by CRP bound to the bacteria [11; 62].

Notably, protection to pneumococcal infection by human CRP-mediated complement activation has been demonstrated in mice, which makes extrapolation to physiologic relevance in humans difficult. Indeed, recently it has been shown that protection to pneumococcal infection by CRP-mediated complement activation does not occur via the classical pathway. Notably, human CRP does not interact with mouse C1q [63]. Thus, the demonstration of a protective role of human CRP in this model remains challenging.

Like CRP, anti-Pc IgM confers protection against pneumococcal infection, which has been demonstrated in mouse models of infection. Immunocomprimised CBA/N mice (xid), which do not produce Abs against a group of thymus- independent antigens, have longer survival times following pneumococcal infection if IgM from normal mouse sera or anti-Pc IgM mAb is administered prior to the challenge [64]. The specificity of these Abs involved in protection is supported by the suppression of T15 *id* with anti-T15, or target deletion of the VH1 gene increases mortality [57,65]. The protective role of anti-Pc IgM is mediated by its

ability to activate the complement system and through interaction with the Fcα/μ receptor. The increased survival after pneumoccal infection in mice conferred by anti-Pc IgM was abrogated after complement depletion with cobra venom factor, demonstrating the crucial role of complement [11,55]. In contrast to IgG and IgE, which interact with FcγR and mediate a wide range of immune responses such phagocytosis, Ab-dependent cytotoxicity, antigen presentation and the production and secretion of many cytokines and chemokines [16] involved in protection, IgM does not interact with these receptors. In vitro experiments have shown that labelled immune complexes composed of IgM and *S. aureus* are endocytosed by B cells. The preincubation of B cells with rat anti-mouse Fcα/μ receptor antibody blocked the endocytosis of the immune complexes. Thus, this receptor appears to mediate endocytosis of IgM-coated bacteria and may be involved in protection [13].

CRP and anti-Pc IgM in cardiovascular disease

Perceptions of CRP as a predictor of coronary heart disease in the healthy population have changed. In publications before 2000, elevated levels of CRP were considered an independent risk factor. In healthy individuals with CRP base-line values higher than 3 mg/L, the relative risk for coronary disease was increased about three-fold [66, 67]. However, recent data show that the predictive value of CRP for coronary disease is lower. After adjustment for other cardiovascular risk factors, such as age, sex, and socioeconomic status, the risk is reduced to 1.45 [68]. The pathophysiogical mechanisms demonstrating association between CRP and coronary disease are not completely clear. Likely, slightly elevated levels of CRP already found in healthy people may be associated with more atherosclerosis, a risk factor for coronary disease [69]. Indeed, atherosclerotic lesions rich in foam cells secrete IL-1 and IL-6, stimuli for CRP production [70,71].

Studies indicate that CRP may have both pro- and anti-atherogenic properties in atherosclerosis [34;72,73]. The anti-atherogenic property is mediated by its ability to activate the complement and to inhibit the OxLDL uptake via scavenger receptors (SRs). The colocalisation of CRP with MAC, enzymatically remodelled LDL, C3d and factor H in the intima of the arterial wall suggests that human CRP might activate the complement in human arteries. The fact that human CRP was deposited in a more extensive area than MAC, suggests that the activation is limited to the early complement components [34,74]. This was confirmed in vitro, since incubation of enzymatically remodelled LDL with human CRP generated substantial amounts of C3b, C3c and C4c, and minimal amounts of the MAC [75]. Thus, lipid particles may be taken up in a non-inflammatory fashion via CR, preventing their capture by the SRs. In addition, CRP inhibits the uptake of OxLDL by SR. SR class A-I/II and CD36 on macrophages are the principal receptors responsible for the uptake of OxLDL [76,77]. It has been shown that the uptake of OxLDL is mediated upon recognition of Pc by CD36. Following the uptake, macrophages transform into foam cells, and release pro-inflammatory cytokines and matrix metalloproteinases, which contributes to atherosclerosis [78]. In vitro, it has been shown that CRP inhibits both the binding and the degradation of OxLDL by murine macrophages and by CD36-transfected cells [79].

The pro-atherogenic property of CRP is explained by the interaction with FcγR. Zwaka *et al*., reported that CRP could mediate the uptake of native LDL through FcγRIIA (CD32) on human macrophages [80]. CRP co-incubated with LDL was added to macrophages in the presence of normal or inactivated serum. Co-localization of CRP, CD32 and LDL was evident exclusively in the presence of normal serum. As mentioned previously, CRP binds

only to modified and not to native LDL [28,81]. Therefore, spontaneous oxidation of LDL by air during the experiments, for example, and not during anti-oxidant conditions, may explain the results of Zwaka *et al*. Thus, CRP cross-linking of FcγR during OxLDL uptake may lead, like cross-linking of the receptor by IgG, to the production of pro-inflammatory cytokines by activated macrophages [16].

Most published studies describe an association of anti-OxLDL Abs and cardiovascular disease. It is known that part of these Abs recognize Pc exposed on the oxidized molecule. An inverse relation between IgM titers to OxLDL and the develop of atherosclerosis has been reported [82,83]. Notably, we found no association between anti-Pc IgM titers at base line and occurrence of AMI in a prospective cohort of healthy people who developed AMI (unpublished Boekhorst, Diaz Padilla *et al*., 2004).

The role of anti-Pc IgM T15 Abs on atherosclerosis has been studied in mouse models [19,37]. Mice deficient for the LDL receptor ($Ldlr^-$) or apoplipoprotein E-null ($Apoe^{-/-}$) mice, which develop extensive atherosclerosis after feeding with a cholesterol-rich diet, have been used. The immunization of cholesterol-fed $Ldlr^-$ mice with a pneumococcal preparation induced high titers of anti-Pc IgM T15, which in turn reduced progression of atherosclerosis [19]. In addition, passive immunization with an anti-Pc IgM mAb reduced accelerated vein graft atherosclerosis in Apoe $^{-/-}$ mice [84].

The anti-atherogenic property is explained by the ability of anti-Pc IgM T15 Ab to block the uptake of OxLDL by macrophages and their transformation into foam cells [37,85]. Pooled plasma from mice immunized with pneumococci, with high levels of Abs to OxLDL, was considerably more effective in blocking the binding of OxLDL to macrophages compared with the plasma from control mice [19]. Thus, once atherosclerotic lesions are induced, the endothelial barrier is destroyed and IgM Abs can gain access to the subintimal space and bind to OxLDL, thus forming immune complexes. The formation of immune complexes in the lumen and in the subintimal space of the blood vessel might block atherogenesis [78], (Figure 5)**.** Significantly increased levels of IgM-OxLDL immune complexes were measured in the plasma of the mice after pneumococcal immunization [16,19].

Anti-Pc IgM T15 is deposited in the atherosclerotic lesions, and their binding to OxLDL may trigger complement activation [19,36,37]. However, studies on complement activation triggered by Abs on the plaques and the role of this mechanism have not been reported.

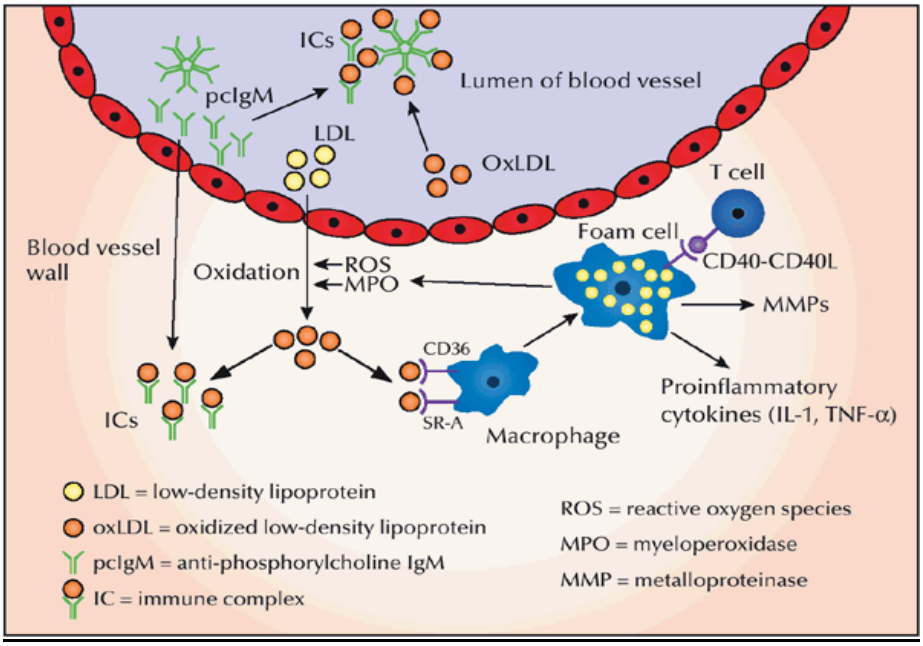

Figure 5. Proposed role for phosphorylcholine-specific autoantibodies in slowing down the progression of atherosclerosis. *Atherosclerotic plaque formation in the blood vessel wall is initiated through uptake of LDL from blood by endothelial cells, and oxidation of LDL by reactive oxygen species within the vessel wall. Uptake of OxLDL by macrophages through scavenger receptors (such as CD36 and SR-A) leads to macrophage transformation into lipid-laden, activated foam cells. In addition, foam cells produce large quantities of myeloperoxidase, an enzyme that produces reactive oxygen species, thereby stimulating oxidation of LDL and further promoting the vicious cycle. The production of IgM-specific Abs increases during atherogenesis (and is enhanced by pneumococcal vaccination). These Abs may be able to cross the endothelial barrier to reach the atherosclerotic lesion. There, they bind to antigens on the surface of OxLDL, forming immune complexes and blocking the uptake of OxLDL by macrophages. In addition, phosphorylcholine-specific IgM may bind OxLDL in the circulation, facilitating its clearance and making it unavailable for plaque formation* [78].
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CRP and natural IgM antibody in ischemia reperfusion injury

Restoration of blood flow (reperfusion) to previously ischemic tissue triggers an inflammatory process, which damages the jeopardized tissue, a phenomenon known as ischemia reperfusion injury (IRI). It is a common pathophysiological event and a source of mortality in clinical conditions such as AMI, stroke, gut ischemia, organ transplantation, and cardiopulmonary bypass. Several inflammatory mediators have been implicated in IRI, including products from polymorphonuclear neutrophilic granulocytes, reactive oxygen species, and complement fragments [25]. Complement activation is a proposed essential step in ischemia/reperfusion-

mediated inflammation, because complement inhibition and complement deficiency considerably attenuate irreversible injury [86-88]. However, the specific complement pathways involved remain unclear. All three complement pathways: the classical, the alternative, and MBL-dependent pathway, may be involved in the development of IRI, depending on the model, the tissue, and the time course of inflammation [89-91]. It has been shown in various clinical settings and in animal models of ischemia and reperfusion that CRP and natural IgM Abs are major mediators of IRI. Part of their deleterious effects has been attributed to their ability to activate the classical pathway of the complement system [91-93].

The involvement of CRP-mediated complement activation to the pathogenesis of disease during AMI has been demonstrated in a rat model. The intraperitoneal injection of human CRP after ligation of the coronary artery enhanced infarction size by 40 % and this enhancement of infarct size was completely prevented by in-vivo complement depletion [93]. In contrast, a recent study described that human CRP injection into rats after occlusion of the middle cerebral artery enhanced infarct size in a non-complement-mediated fashion [94]. Systemic complement depletion with cobra venom factor in these rats did not affect cerebral infarct size, indicating that circulating complement does not contribute to injury in this model. It could be suggested that another "pathogenic mechanism" of CRP may be involved in this increase. However, the increase in infarct size observed in the treated CRP animals showed a borderline difference compared with the albumin-treated rats, making it difficult to draw a conclusion from this study.

In human myocardial infarction as well as in the AMI rat model, colocalization of CRP with complement was observed in ischemic tissue [95]. This initial finding suggested that human CRP could activate the complement system in human AMI similar to its role in the experimental model. A further study demonstrated that human CRP is able to activate locally the complement system, since CRP-complement complexes, specific makers for CRP activation, were measured in homogenates prepared from infarcted human myocardium [96]. However, in humans no correlation between the extent of CRP and complement deposition and infarct size has been found [97]. So far, it is not known to what extent CRP contributes to infarct size in humans.

The development of non-complement-activating Abs against Pc that prevent the binding of CRP to the ischemic cells may provide further insight in the role of CRP and may also provide a therapy for reducing IRI. Human mAb of the IgG4 subclass could be useful in humans for therapeutic purposes because they are unable to significantly activate the complement system [16]. The cross-reactivity of such Abs with vital cells must be investigated. Furthermore, modified CRP molecules, able to bind to the appropriate ligand on the ischemic cells but not to activate complement, could provide another therapeutic approach in reducing injury after ischemia and reperfusion. Observations in mouse models have revealed that natural IgM Abs may be involved in the pathogenesis of IRI [86,91,98-100]. The identification of the neo-antigen(s) exposed or expressed by the ischemic cells and the specificity of IgM are still under investigation. Initial experiments with mice that lack all classes of Abs, RAG-1 $^{-/-}$ and RAG- 2 $^{-/-}$, have revealed that these mice develop less IRI than wild-type mice. The administration of normal mouse sera and/or purified IgM to these mice in a hind limb or intestinal model of IRI, respectively, restored the damage to the levels observed in wild-type mice [86,91]. Following reconstitution, IgM and C3 were deposited on the endothelium of the ischemic reperfused mice but absent in sham controls. Knockout mice deficient in C4 ($C4^{-/-}$) were found to be equally protected from local injury as were mice deficient in C3 ($C3^{-/-}$) in both models of ischemia and reperfusion. These data demonstrate that the dependence on complement is via the classical pathway and support the role of natural Abs- mediated injury.

Further studies with CR2-deficient ($CR2^{-/-}$) mice confirmed natural IgM to be involved in the reperfusion injury. These mice lack CR2, resulting in a decreased repertoire of $CD5^{+}$B-1 cells, and they are protected from IRI. Indeed, infusion with natural IgM purified from B-1 cells restored the injury to the levels observed in the wild-type mice in models of intestinal IRI. In contrast, reconstitution with pooled murine IgG alone did not restore histological injury but enhanced neutrophil infiltration when combined with IgM. C3 was exclusively deposited in the intestines of mice subjected to ischemia and reperfusion and treated with IgM. Therefore, IgM was the most potent complement-fixing isotype in this model, and responsible for IRI [101].

More recently, a mAb IgM from a specific clone (CM-22; IgM^{CM-22}) of B-1 cells has been described that can restore injury in mice subjected to skeletal and intestinal IRI [99,100]. A protein of high molecular mass of about 250 KDa co-precipitated with IgM^{CM-22}. Analysis of this high-molecular-mass band yielded peptide sequences homologous to nonmuscle myosin heavy chain (NMHC) type II A and C. A highly conserved region within both isoforms of NMHC has been identified as the target for IgM $^{CM-22}$ and initiation of the injury in murine models of skeletal and intestinal ischemia and reperfusion. A synthetic peptide of 12 amino acids, representing the conserved region of NMHC was synthesized (P8). P8 bound IgM^{CM-22} in vitro and blocked IRI in the two distinct tissues. Furthermore, IgM^{CM-22} and C4 colocalized in the intestinal tissue after ischemia and reperfusion, and this deposition correlated with the pathology. It indicates that the pathogenic property of this IgM is mediated by its ability to activate the classical pathway of complement [100].

Conclusions

CRP and anti-Pc IgM, two structurally different proteins of the innate immune system, share the ability to bind to Pc in heterologous and altered self-molecules. Both proteins are able to protect against infections, likely through their ability to activate the complement system via the classical pathway. The protective role of human CRP has been demonstrated in heterologous systems. Surprisingly, it has recently been reported that human CRP does not interact with mouse C1q. Further studies with mouse, rabbit and rat CRP in autologous systems are warranted to elucidate the role of complement in protection against infection in this system. There is evidence for a beneficial role of CRP and anti-Pc IgM in atherosclerosis. The binding of IgM and CRP to Pc residues on OxLDL blocks its uptake by SR on macrophages. This may prevent the transformation of macrophages into lipid-laden, activated foam cells, which promote the pro-inflammatory milieu in the atherosclerotic plaques. Studies on depositions of complement proteins suggest that CRP-mediated complement activation leads to reduced generation of MAC, which may also be beneficial. Further studies should shed more light on the role of CRP and anti-Pc IgM-mediated complement activation and atherogenesis. It seems that both proteins have survived in the evolution by the two actions mentioned above: protection against infection by Pc-containing microorganisms and clearance of oxidatively altered self-molecules. However, considerable evidence now points to an important role for CRP and natural IgM Ab in the pathogenesis of IRI. Ischemia leads to the exposition of “altered phospholipids” and new proteins on the cell membranes, which are recognized by plasma proteins such as CRP and natural IgM Ab. In various clinical settings and in animal models of ischemia and reperfusion, it has been demonstrated that complement activation by CRP and natural IgM Ab are major mechanisms of IRI.

Scope of the thesis

The scope of this thesis is to assess the roles of CRP-, IgM- and anti-Pc-IgM-mediated complement activation in tissue damage, in particular in IRI. The objective is to provide more insight into the relative contributions of these proteins in this process. Chapter 2 reports a study on the capability of rCRP to activate rat complement and to mediate IRI. It provides experimental evidence that rat CRP is indeed able to activate the rat complement system upon ligand binding. This indicated that rat models are appropriate for studying the role of CRP in IRI. Chapter 3 concerns a study in rats. It reports that, although rat CRP levels increase upon estrogen replacement, this does not result in complement activation because there is not in generation of CRP ligands in this model. Chapter 4 deals with the role of CRP, IgM and anti-Pc IgM in relation to complement activation in a rat model of intestinal IRI. The effect of C1-inhibitor (C1-Inh) on inflammatory mediators was also studied. The study demonstrated that CRP, IgM and complement are deposited in the intestine after IRI. Furthermore, C1-Inh either administered before or at the end of ischemia reduced complement activation, indicating that it may have therapeutic benefits. Chapter 5 deals with the question whether or not anti-Pc IgM, like CRP, is a cardiovascular risk marker. For this study, an immunoassay was developed for the quantitation of anti-Pc IgM. In healthy donors and in patients with skin tumors undergoing isolated limb perfusion with tumor necrosis factor alpha, the relation between levels of anti-Pc IgM and CRP was investigated. The results suggest that anti-Pc IgM and CRP constitute two independent mechanisms involved in the elimination of injured cells. Chapter 6 is devoted to the relationship between IgM binding to apoptotic cells and infarct size in patients with AMI. The findings suggest that anti-Pc IgM levels may modify inflammatory responses and infarct size in patients with AMI.

Based on the literature and our own findings we hypothesize that during tissue damage as seen in ischemia and reperfusion, neoepitopes are exposed in the membranes of damaged cells. One of these neoepitopes could be Pc, the head group of lysophosphatidylcholine exposed after flip-flop of the membrane, oxidation and hydrolysis of the phospholipids by phospholipases A_2. CRP and IgM may bind to Pc, and this binding may be followed by complement activation via the classical pathway. Because damaged tissue lacks regulatory molecules that in normal tissues prevent excessive complement activation, complement proteins may attack reversibly injured cells, which then become irreversibly damaged. Furthermore, we propose that the relative contribution of these proteins to complement activation after ischemia reperfusion varies between species, depending on their baseline values. For instance, in animals with high CRP levels and low IgM, as in rats, CRP will be the dominant activator of complement following IRI.

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CHAPTER 2

Rat C-reactive protein activates the autologous complement system

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Rat C-reactive protein activates the autologous complement system

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SUMMARY

Activation of complement is a biological function of human C-reactive protein (hCRP), whereas rat CRP (rCRP) has been claimed to be unable to activate complement. As important biological functions of proteins are probably conserved among species, we re-evaluated, using various ligands, the capability of rCRP to activate complement. The activation of complement by hCRP and rCRP was investigated in solid- and fluid-phase systems. In the solid-phase system, purified CRP was fixed to enzyme-linked immunosorbent assay (ELISA) plates and incubated with human or rat recalcified plasma. Dose-dependent binding of human and rat C3 and C4 was observed to human and rat CRP, respectively. In the fluid-phase system, recalcified rat plasma, which contains about 500 mg/l of CRP, or human plasma supplemented with hCRP, were incubated with lyso-phosphatidylcholine. A dose-dependent activation of complement was observed upon incubation with this ligand, as reflected by the generation of activated C4 as well as of CRP–complement complexes. This activation was, in both cases, inhibited by preincubation of plasma with p-aminophosphorylcholine, a specific inhibitor of the interaction of CRP with its ligands, or by chelation of calcium ions. We conclude that rat CRP, similarly to human CRP, can activate autologous complement. These results support the notion that opsonization of ligands with complement is an important biological function of CRP.

INTRODUCTION

C-Reactive protein (CRP) is a pentraxin found in most vertebrates (e.g. mice, rats and humans) and in invertebrates such as the horseshoe crab (*Limulus polyphemus*).[1–3] Human CRP (hCRP), discovered because it binds to pneumoccocal C-polysaccharide (CPS),[4] is an acute-phase protein, of which plasma levels can increase up to 1000-fold following tissue damage or infection. CRP also binds to phosphorylcholine (PCh), which amongst others is found in membrane phospholipids. Among the effector functions exerted by CRP upon binding to ligands, is activation of complement. This activation proceeds via the classical pathway and occurs *in vitro*,[2,5] as well as *in vivo*.[6] Opsonization of its ligands with complement fragments is probably an important function of CRP.

In rats, CRP (rCRP) is not a typical acute-phase protein.[7] However, in contrast to humans, rats have much higher plasma CRP concentrations under basal conditions, i.e. about 300–500 mg/l, which is 100 times higher than the concentration in humans.[8] Remarkably, in contrast to hCRP, rCRP has been reported to be unable to activate complement,[9,10] despite the fact that hCRP and rCRP share 70% amino acid homology.[11] Complement activation by rCRP has only been studied using the C-polysaccharide of *Streptococcus pneumoniae*.[9] Hence, it is possible that rCRP can activate complement upon binding to other ligands.

We recently developed assays for complexes of CRP and activated complement fragments, and showed that these

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Abbreviations: AHG, aggregated human IgG; CPS, pneumococcal C-polysaccharide; CRP, C-reactive protein; hCRP, human C-reactive protein; NHP, normal human plasma; NRP, normal rat plasma; PC, phosphatidylcholine; PCh, phosphorylcholine; PBS, phosphate-buffered saline, pH 7·4; PBS-T, PBS containing 0·02% (wt/vol) Tween-20; PTG, PBS-T containing 0·2% (wt/vol) gelatin; poly-HRP, streptavidin-polymerized horseradish peroxidase; rCRP, rat C-reactive protein; strept-PO, streptavidin coupled to monomeric peroxidase; VB^{2+}, veronal-buffered saline containing 5 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7·4.

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complexes specifically reflect CRP-mediated complement activation.[6] Moreover, significant levels of CRP–complement have been found in plasma samples, indicating that hCRP can activate complement *in vivo*. In the present study we investigated the binding of rat complement fragments to solid-phase bound CRP. In addition, we developed an assay for complexes between rat C3 and rCRP, and used this assay to re-evaluate the capability of rCRP to activate the complement system. Our results indicate that rCRP can activate autologous complement and support the hypothesis that activation of complement upon binding to a suitable ligand is an important biological function of this pentraxin.

MATERIALS AND METHODS

Materials

Cyanogen bromide (CNBr)-activated Sepharose 4B and Sephadex® G25 Fine were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). PCh, the calcium salt of PCh chloride, and 1-α-lyso-phosphatidylcholine (lyso-PC) were obtained from Sigma Chemical Co. (St Louis, MO). CPS was obtained from Statens Serum Institut (Copenhagen, Denmark). The molecular weight (MW) protein standard, 10-kDa protein ladder (10 000–200 000 MW) was from GibcoBRL, Life Technologies, Inc. (Gathersburg, MD). Streptavidin coupled to polymerized horseradish peroxidase (poly-HRP) was obtained from the Business Unit Immune Reagents of our institute (Sanquin). Streptavidin coupled to monomeric peroxidase (strept-PO) was purchased from Amersham Pharmacia Biotech. L-C-biotin-N-hydroxysuccimide ester was from Pierce Chemical Co. (Rockford, IL).

Plasma samples

Recalcified normal rat plasma (NRP) was prepared from rat blood collected in 10 mM EDTA. The blood was centrifuged for 10 min at 1300 ***g***. The supernatant was then supplemented with $CaCl_2$ to a final concentration of 12 mM. After incubation for 20 min at 37°, the fibrin clot was removed by centrifugation at 1300 ***g*** for 15 min at 4°. Finally, the recalcified plasma was stored in aliquots at −70° until required for use in activation experiments. Recalcified normal human plasma (NHP) was obtained in a similar manner.

Antibodies and proteins

Rabbit serum containing polyclonal antibodies against hCRP (KH61) was obtained from the Business Unit Immune Reagents of our institute. Mouse monoclonal antibodies (mAbs) against human C3d or C4d were as previously described.[12] Notably, these antibodies also react with C3b and C3bi, and C4b and C4bi, respectively. Antibodies were purified using affinity chromatography on protein G–Sepharose (Pharmacia Fine chemicals, Uppsala, Sweden) according to the manufacturer's instructions, and biotinylated using standard procedures. The mouse mAb against rat C3/C3b/bi has been described previously.[13] Human C1q was purified as described previously[14] and biotinylated according to the instructions provided by Pierce. Aggregated human immunoglobulin G (AHG) was prepared as described previously.[15]

Purification of rat and human CRP

rCRP was purified from rat plasma collected in 10 mM EDTA from healthy Wistar rats. Ascites fluid, therapeutically collected from carcinoma patients, was used as a source for human CRP. For the purification of rat and human CRP, 4 vol of recalcified plasma, or ascites, were mixed with 1 vol of 5× binding buffer [2·5 M NaCl, 50 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Veronal buffer, 0·5% (wt/vol) Tween-20, 0·5% sodium azide (all final concentrations) pH 7·4] and applied onto PCh bound to Sepharose. The PCh–Sepharose had been equilibrated with binding buffer [0·5 M NaCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Veronal buffer, 0·1% (wt/vol) Tween-20, 0·02% (wt/vol) sodium azide]. After incubation overnight at 4°, the gel was washed with binding buffer until the absorbance at 280 nm was $<0{\cdot}04$. Bound proteins were then eluted with phosphate-buffered saline, pH 7·4 (PBS), containing 10 mM EDTA. The collected fractions were dialysed against PBS and stored at 4°, until analysed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; see below).

Preparation of rabbit antibodies against rCRP

rCRP was eluted from PCh–Sepharose using a PCh gradient (0–2 mM), as described previously.[16] The fractions containing rCRP were pooled and subjected to preparative electrophoresis. Proteins were separated by SDS–PAGE (4–12% gel) and blotted onto polyvinylidene difluoride (PVDF) membrane, 0·45-μm pore size (Millipore Corporation, Bedford, MA). Two bands of 24 000 and 50 000 MW were excised, eluted as described previously,[17] and used as immunogens.

Rabbits were immunized by intramuscular injection of 50 μg of purified rCRP emulsified in Freund's complete adjuvant (Difco Laboratories, Amsterdam, the Netherlands), followed by three booster injections of 50 μg of purified rCRP in Freund's incomplete adjuvant. Affinity chromatography was used to purify specific antibodies from the rabbit antisera. Four milligrams of rCRP was coupled to CNBr-activated Sepharose 4B, according to the manufacturer's instructions. The gel was then incubated with the antiserum, washed and layered on top of a Sephadex® G25 Fine gel-filtration column equilibrated in PBS. Bound antibodies were then eluted with 0·2 M glycine, pH 2·8. To remove contaminating antibodies, the affinity-purified anti-rCRP antibodies were subsequently absorbed with CRP-depleted rat plasma coupled to CNBr-activated Sepharose beads. CRP-depleted rat plasma was prepared by absorbing recalcified rat plasma onto PCh–Sepharose in the presence of 0·5 M NaCl.

One milligram of purified rabbit anti-rat CRP was biotinylated, using standard procedures, and tested for specificity in immunoblotting. NRP, or CRP-depleted rat plasma was electrophoresed on SDS polyacrylamide gels under non-reducing conditions, as described above. The proteins were then transferred onto PVDF membranes, 0·2-μm pore size (Novex, San Diego, CA).[18] The membranes were then incubated for 1 hr in PBS containing 4% (vol/vol) cow's milk and subsequently for 90 min with biotinylated anti-rCRP, diluted 1 : 500 in PBS containing 0·4% (vol/vol) of milk and 0·1% (wt/vol) Tween-20 (J. T. Baker, Deventer, Holland). After washing in the same buffer, the membranes were incubated for 1 hr with strept-PO (diluted 1 : 500 also in the same buffer) and washed. Peroxidase

was visualized by staining using the enhanced chemiluminescence (ECL) technique (Amersham-Pharmacia).

General procedure for enzyme-linked immunosorbent assays (ELISAs)

Nunc, Maxisorp plates (Roskilde, Denmark) were incubated overnight at room temperature with protein (antibodies or CRP) diluted in 0·1-M carbonate/bicarbonate buffer, pH 9·6. The final volume of this and the other steps was 100 µl. After washing the plates twice with PBS, residual binding sites were blocked for 1 hr at room temperature with PBS containing 2% (vol/vol) cow's milk. The plates were then washed with PBS containing 0·02% (wt/vol) Tween-20 (PBS-T). Samples to be tested were then incubated in the indicated buffer for 1 hr at room temperature. Plates were then washed with PBS-T and incubated with strept-PO, diluted 1 : 1000, or with poly-HRP, diluted 1 : 10 000, in PBS containing 2% (vol/vol) milk to block non-specific binding. Finally, plates were washed and the peroxidase activity was visualized by incubation with 3,3′,5,5′,-tetramethyl-benzidine (TMB), 100 µg/ml in 0·11 M sodium acetate, pH 5·5, containing 0·003% (vol/vol) H_2O_2. The reaction was stopped after 10 min by the addition of 2 M H_2SO_4, and the absorbance at 450 nm was measured using a Titertek Multiscan plate reader (Flow Laboratories, McLean, VA).

ELISA for rCRP

Purified rabbit antibodies against rCRP (1 µg/ml) were used as catching antibodies. The samples to be tested were serially diluted in PBS containing 0·1% (wt/vol) Tween 20 and 0·2% (w/v) gelatin (PTG). Biotinylated anti-rCRP were diluted in ELISA-ultraperformance buffer supplemented with 10 mM EDTA. Plates were developed with strept-PO. Purified rCRP was used as a standard.

ELISA for activated rat C4

Activation of rat C4 was detected as described previously.[19] Results were expressed as percentage of the maximal amount of activated C4 generated in rat serum by incubation with purified C1s (Calbiochem, La Jolla, CA). As the assay does not discriminate between C4b, C4bi or C4c, the activation product detected was referred to as C4b/c.

ELISA for rat CRP–C3 complexes

An in-house standard was prepared by incubating NRP with lyso-PC at 800 µg/ml, for 1 hr at 37°, and absorbing the mixture onto PCh–Sepharose for 3 hr at 4°. The Sepharose beads were washed extensively with veronal buffer containing 0·65 M NaCl, 20 mM $CaCl_2$, 4 mM $MgCl_2$ and 0·1% (wt/vol) Tween-20. CRP and CRP–complement complexes were then eluted from the Sepharose beads with PTG-25 mM EDTA, overnight at 4°. Samples to be tested were also absorbed onto PCh–Sepharose, using a similar procedure, except that they were not incubated with lyso-PC. The eluates of samples were stored at −70° until required for testing.

In the ELISA, rat C3 mAb was used as catching antibody. The samples to be tested, i.e. the eluates from the PCh–Sepharose (see above), were diluted in PTG containing 25 mM EDTA. Binding of CRP–C3 complexes was visualized with biotinylated rabbit anti-rCRP, as described above for the ELISA for rCRP. Bound anti-CRP was measured using poly-HRP.

Binding of biotinylated human C1q to solid-phase rCRP or hCRP

Rat or human CRP was incubated overnight in 0·1 M carbonate/bicarbonate buffer, pH 9·6, in ELISA plates. Incubation volumes were 100 µl. After blocking residual binding sites with 0·1% (wt/vol) bovine serum albumin (BSA) in PBS, the plates were washed with 50 mM NaCl, 20 mM Tris (pH 7·4), 10 mM $CaCl_2$ and 0·1% Tween-20, and incubated for 1 hr at room temperature with biotinylated C1q diluted to 0·0625–50 µg/ml in the same buffer. After five washes with Tris buffer, the plates were incubated with poly-HRP diluted 1–10 000 in PBS containing 2% milk, for 20 min at room temperature. Finally, peroxidase activity was visualized and measured as described above.

Binding of rat C1q to solid-phase rCRP or hCRP

Different concentrations (0·25–10 µg/ml) of rCRP or hCRP were coated onto ELISA plates as described above. The plates were then washed twice with 50 mM NaCl, 20 mM Tris (pH 7·4), 10 mM $CaCl_2$, 0·1% (wt/vol) Tween-20, and incubated with 0·1% (wt/vol) BSA in PBS to block residual binding sites. After one wash, serial dilutions of NRP in Tris (pH 7·4) containing 0·1% Tween-20, were incubated in the plates for 1 hr at room temperature. As a control, inactivated rat plasma (30 min, 56°) was also tested. Finally, the plates were incubated with rabbit anti-rat C1q in Tris–Tween buffer. Bound rabbit antibodies were detected by a subsequent incubation with poly-HRP, as described above.

Deposition of rat or human C3 on solid-phase rCRP or hCRP

To assess the complement-activating capability of CRP bound to a solid phase, ELISA plates were coated with 10 µg/ml of rat or human CRP, as described above. After this and the other incubations the plates were washed with PBS containing 0·1% (wt/vol) Tween-20. Residual binding sites on the plates were blocked by incubation for 1 hr at room temperature with BSA (0·1%, wt/vol) in PBS. After five washes, the plates were incubated for 1 hr at 37° with recalcified NHP or NRP appropriately diluted in veronal-buffered saline containing 5 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7·4 (VB^{2+}), containing 0·1% (wt/vol) Tween-20. The deposition of rat or human C3b/c and C4b/c was detected by incubation, for 1 hr at room temperature, with biotinylated antibodies against rat or human C4b/c or C3b/c, respectively. Finally, the plates were incubated with strept-PO (diluted 1 : 1000) for 30 min at room temperature. The peroxidase activity was visualized with TMB, as described above.

Complement activation by hCRP in the fluid phase

Recalcified NHP (50 µl) (1 vol) was incubated for 1 hr at 37° with 1 vol of rat or human CRP (4, 20 or 50 µg/ml) in VB^{2+}, 1 vol of VB^{2+} containing 800 µg/ml of lyso-PC, and 1 vol of VB^{2+}. CRP (1 vol) preincubated for 1 hr with PCh (20 mM in 1 vol of VB) was used as a negative control. Then, 10-µl samples of the incubation mixtures were tested for complement-activation products in the ELISA for human C4b/c and C3b/c, respectively, as described by Wolbink *et al.*[6]

Complement activation by rCRP in the fluid phase

To study activation of the autologous complement system by native rCRP, 1 vol of recalcified NRP (containing 492 μg/ml CRP) was added to 1 vol of VB^{2+} containing different concentrations (0·1–0·8 mg/ml) of lyso-PC, and 2 vol of VB^{2+}. The mixtures were then incubated for 1 hr at 37°. As a control, a similar procedure was performed, except that VB^{2+} containing 1 M NaCl, rather than VB^{2+}, was used. As another negative control, NRP (1 vol) was incubated with 1 vol of lyso-PC, in the presence of PCh (20 mM in 1 vol of VB^{2+}). The mixtures were then tested for C4b/c generation and for the presence of rCRP–C3 complexes. The effect of CPS on CRP-mediated complement activation in rat plasma was tested in a similar manner. To assess the specificity of complement–CRP complexes for CRP-induced complement activation, NRP was also incubated with AHG (1·25 mg/ml, final concentration) and tested for CRP–C3 complexes and generation of C4b/c.

RESULTS

Purification and characterization of CRPs

Rat and human CRP were purified by affinity chromatography using PCh–, as described above in the Materials and methods. The apparent MW of both hCRP and rCRP was estimated on SDS–PAGE (data not shown). rCRP migrated under non-reducing conditions as two bands of 24 000 and 50 000 MW, whereas hCRP migrated as one band of 21 000 MW. Under reducing conditions, rCRP migrated as one band of 27 000 MW and hCRP as one band of 23 000 MW. The observed migration patterns fitted well with published data.[7,9,16]

Specificity of anti-rCRP

Rabbits were injected with rCRP purified by preparative SDS–PAGE, as described above in the Materials and methods. Specific antibodies were purified using affinity chromatography with CRP–Sepharose and absorbed onto CRP-depleted NRP coupled to Sepharose. The absorbed fraction was then biotinylated according to standard procedures. The specificity of the rabbit polyclonal anti-rCRP, purified in this way, was then analysed by Western blot. The antibodies reacted with 24 000-MW, 50 000-MW and high-MW bands of rCRP in the NRP. Similar bands were observed when purified CRP was tested (not shown). The high-MW bands correspond well with those observed by others in purified rCRP preparations.[9] Depletion of NRP for CRP by adsorption onto PCh–Sepharose, completely abolished the reactivity of anti-rCRP with the depleted plasma. Hence, we concluded that the rabbit polyclonal antibodies against rCRP were specific, reacting with rCRP monomers and dimers. The specificity of the anti-rCRP was further supported by the observation that in double immunodiffusion, the antiserum was found to be completely identical to an antiserum against rat CRP, kindly provided by Dr M. B. Pepys (data not shown).

ELISA for rCRP

Using the polyclonal antibodies described above, an ELISA for rCRP was developed. The assay appeared to have a sensitivity of about 5 ng/ml rCRP, as assessed using purified rCRP. Figure 1(a) shows the dose–response curves of NRP and NRP depleted for CRP. Absorption onto PCh–Sepharose caused a shift of the dose–response curves of about 1000-fold, indicating that about 0·1% of rCRP was not removed by this procedure. The substitution of the catching anti-rCRP by rabbit immunoglobulin G (IgG) completely abrogated the response of purified rCRP or NRP in the ELISA, as did substitution of the biotinylated anti-rCRP with biotinylated normal rabbit IgG (Fig. 1b). These results demonstrated the specificity of the ELISA. The intra-assay coefficient of variation was less than 11% and that of the interassay coefficient was less than 16%.

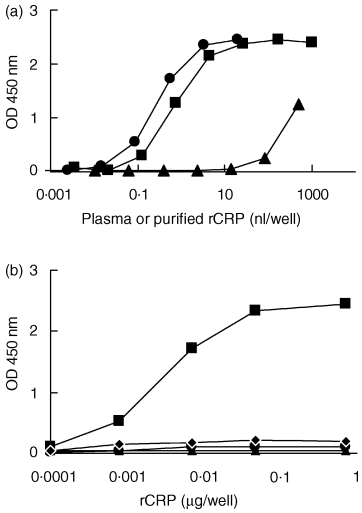

Figure 1. Enzyme-linked immunosorbent assay (ELISA) for rat C-reactive protein (rCRP). (a) Dose–response curves of normal rat plasma (■), depleted rat plasma (▲), or purified CRP (●). (b) A control experiment in which the catching anti-rCRP or the detecting biotinylated anti-rCRP was replaced with normal rabbit immunoglobulin G (IgG) (▲) or biotinylated normal rabbit IgG (●), or both (◆). Purified rat CRP was tested in each of these ELISAs. Results obtained with the normal ELISA are also shown (■).

Complement activation by solid-phase CRP

Rat and human CRP bound biotinylated human C1q. This binding was dose dependent for both C1q and CRP (Fig. 2). Purified rat C1q was not available. Binding of rat C1q to rCRP was tested in a different way, by incubation of CRP-coated plates with different dilutions of NRP. C1q binding was then assessed with biotinylated rabbit anti-rC1q. In this assay, rC1q appeared to bind to rCRP (see Fig. 3). Incubation of the rat plasma for 30 min at 56° completely abolished the binding of C1q (data not shown). Next, we investigated the deposition of rat or human C3b onto either rCRP or hCRP fixed onto the solid phase. Upon incubation with NRP or NHP, and using biotinylated mAbs against human C3b/c or rat C3b/c, deposition of activated complement factors on rCRP or hCRP was

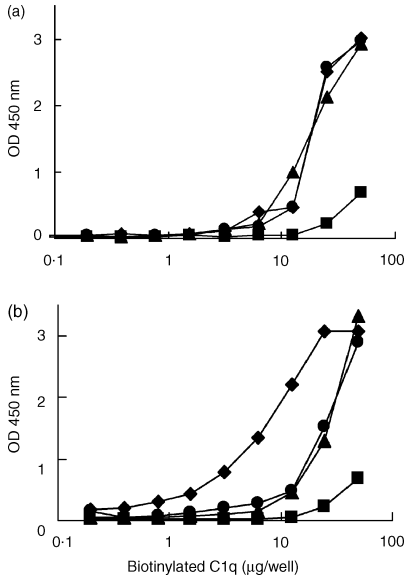

Figure 2. Binding of human C1q to human (a) or rat (b) C-reactive protein (CRP) fixed onto an enzyme-linked immunosorbent assay (ELISA) plate. CRP, at different concentrations (10 μg/ml, ◆; 1 μg/ml, *; 0·5 μg/ml, ▲; or buffer, ●), was coated onto ELISA plates. The coated plates were incubated with different dilutions (0·0625 to 50 μg/ml) of biotinylated human C1q. After incubation with streptavidin-polymerized horseradish peroxidase, the peroxidase activity was visualized with 3,3′,5,5′,-tetra-methyl-benzidine (TMB).

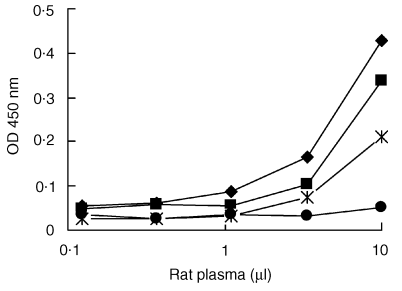

Figure 3. Binding of rat C1q to rat C-reactive protein (rCRP). Different concentrations of rCRP (10 μg/ml, ◆; 1·25 μg/ml, ■; or 0·08 μg/ml, *) or buffer (●) were coated onto enzyme-linked immunosorbent assay (ELISA) plates and incubated with rat plasma. Bound C1q was detected with rabbit anti-rat C1q.

observed (Fig. 4). This deposition was completely abolished in the presence of 20 mM EDTA, indicating that deposition was caused by an activation process and not by non-specific binding of activated complement components to the CRP-coated plates.

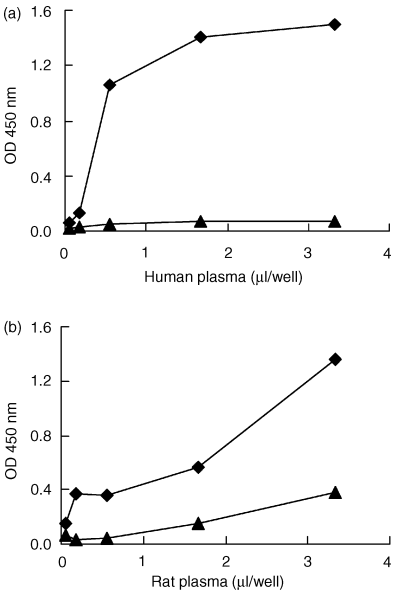

Figure 4. Fixation of human or rat C3b on human or rat C-reactive protein (CRP) onto an enzyme-linked immunosorbent assay (ELISA) plate. ELISA plates were coated with human (a) or rat (b) CRP at 10 μg/ml (◆), or with buffer (▲), and incubated with increasing volumes (0 to 3·33 μl) of human or rat plasma. Then, C3b/c fixation on CRP was detected with specific monoclonal antibody (mAb), as described in the Materials and methods. This figure is representative of three experiments, with similar results obtained on each occasion.

Complement activation by fluid-phase CRP

The incubation of recalcified NHP with 50 μg/ml of rCRP or hCRP in the presence of lyso-PC (400 μg/ml) yielded maximal generation of human C3b/c and C4b/c, as measured by ELISA. This generation was blocked when hCRP or rCRP was pre-incubated with 20 mM PCh. Incubation of recalcified NHP with lyso-PC alone did not generate a significant amount of activated complement factors. These results thus demonstrate that rCRP is able to activate complement in human plasma. To assess whether it can also activate endogenous complement in rat plasma, we incubated rat plasma with different concentrations of lyso-PC. As rat plasma contains approximately 0·5 mg/ml of CRP, we did not add purified CRP to this plasma. As described in Table 1, a dose-dependent activation of C4 was observed upon addition of lyso-PC to rat plasma. This activation was abrogated in the presence of high salt or PCh, a low-molecular-weight ligand for CRP, confirming that the observed activation was caused by CRP.

We have previously demonstrated that activation of human complement by hCRP results in the generation of measurable amounts of complexes between CRP and activated C3 or C4.[6] To further substantiate activation of rat complement by rCRP, we developed an ELISA for the quantification of rat complement–CRP complexes in plasma. When NRP, incubated with

Table 1. C-reactive protein (CRP)-dependent activation of complement in rat plasma *in vitro*

Activator (mg/ml)	CRP–C3 (U/ml)	C4b/c (% NRA)
lyso-PC		
0·8	693·0 (±32)	15·2 (±0·4)
0·4	473·0 (±52)	11·2 (±4·4)
0·2	276·0 (±40)	10·2 (±2·2)
0·1	168·0 (±7)	9·4 (±2·8)
lyso-PC 0·8 + PCh	31·0 (±0·54)	3·7 (±0·2)
lyso-PC 0·8 + NaCl	10·7 (±0·56)	2·3 (±0·9)
CPS		
0·250	93·9 (±3·2)	13·2 (±4)
0·125	126·0 (±11)	14·5 (±4·5)
CPS 0·125 + PCh	30·9 (±0·6)	5·1 (±2·4)
CPS 0·125 + NaCl	ND	2·1 (±0·5)
Controls		
Buffer (60 min, 37°)	52·0 (±8)	8·6 (±0·3)
Buffer (on ice)	3·0 (±0·22)	1·2 (±0·8)
AHG (1·25 mg/ml)	9·0 (±0·44)	43·3 (±0·4)
Trypsin (0·25 mg/ml)	19·0 (±3)	13·0 (±4·6)

Normal rat plasma was incubated with lyso-phosphatidylcholine (lyso-PC) or pneumococcal C-polysaccharide (CPS), as described in the Materials and methods. The concentration of C4b/c and CRP–C3 complexes was then measured. The results represent the mean ± standard error of the mean (SEM) of three separate experiments. Generation of C4b/c and of complexes by either activator was inhibited by 10 mM EDTA (data not shown), 20 mM phosphorylcholine (PCh), or 1 M NaCl (NaCl).

AHG, aggregated human IgG; ND, not determined.

800 μg/ml of lyso-PC, was tested in this assay, a significant dose–response was observed, whereas no complexes were detected in NRP (Fig. 5). Substitution of the capture antibody (anti-rat CRP) or the detecting antibody with an irrelevant mAb or biotinylated normal rabbit IgG, respectively, abolished the response observed in the ELISA (results not shown). As shown in Table 1, the generation of CRP–C3 complexes in rat plasma by lyso-PC was dose dependent and inhibited in the presence of 1 M NaCl (which prevents binding of C1q to CRP) or PCh.

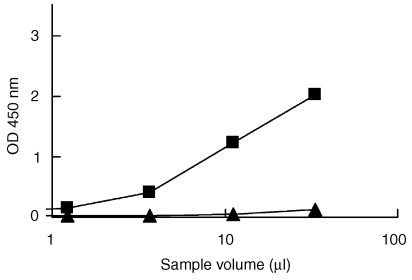

Figure 5. Enzyme-linked immunosorbent assay (ELISA) for rat C-reactive protein (CRP)–C3 complexes. Phosphorylcholine (PCh)–Sepharose eluates of normal rat plasma (▲) or rat plasma incubated with lyso-phosphatidylcholine (lyso-PC) (■) were prepared as described in the Materials and methods, and tested in the ELISA.

When CPS was used to activate rat complement via the CRP, results similar to those obtained with lyso-PC were observed, although somewhat fewer complexes were generated (Table 1). In contrast, no CRP–C3 complexes were generated when rat plasma was incubated with aggregated IgG, although this activator induced a marked activation of C4 (Table 1). Similarly, incubation of rat plasma with trypsin also did not generate CRP–C3 complexes.

DISCUSSION

The ability of ligand-bound CRP to activate the complement system seems to be a conserved function of this protein.[2,6,9,20–22] A remarkable exception, however, is rat CRP, which, based on studies with CPS as a ligand, was found to be unable to activate complement.[9,10] In the present study we demonstrate, using various approaches and various ligands, that rCRP can activate complement.

We isolated rCRP using PCh coupled to Sepharose. SDS–PAGE revealed a migration pattern similar to that described in the literature.[7,9,16] The apparently higher MW of the monomeric subunit under reducing conditions has also been described previously and is suggested to be a result of the existence of an intrachain disulphide bridge.[7,9,23] Hence, we concluded that the protein purified from rat plasma met the criteria previously described for rCRP.

Human C1q binds only to aggregated or complexed CRP to initiate complement activation, and does not bind to monomeric CRP.[24,25] In our experiments, both rat and human CRP were aggregated onto ELISA plates. This method of using protein-coated surfaces for studying complement fixation has been used previously, for example, in studies of complement activation by IgG or immunoglobulin M (IgM), defensins or C3-binding glycoprotein.[26–29] rCRP fixed onto an ELISA plate bound human C1q, in agreement with observations by Baltz *et al.*,[23] suggesting that it can activate human complement. rCRP on the ELISA plate also fixed rat C1q, as did C3 upon incubation with recalcified rat plasma; notably, fixation was not observed in the presence of EDTA. Thus, we concluded that rCRP fixed onto an ELISA plate can activate the rat complement system.

It could be argued that the affinity purification of rCRP, or the fixation of this protein onto an ELISA plate, induces conformational changes leading to complement-activating properties that do not reflect the changes induced by binding to natural ligands. Therefore, we also studied activation of complement by native CRP interacting with ligands in rat plasma. The CRP baseline level in rats is about 0·5 g/l.[8,30–32] Hence, we studied activation of complement via CRP by incubating NRP with different CRP ligands, without additional CRP supplementation. Addition of lyso-PC or CPS induced the generation of C4b/c in rat plasma, which was inhibited by EDTA and also by p-amino-phenylphosphorylcholine, a monovalent ligand for CRP that prevents its aggregation on multivalent ligands. These results strongly suggested that native CRP in rat plasma could activate complement upon incubation with appropriate ligands.

In previous studies we demonstrated that the formation of human CRP–complement complexes, i.e. complexes of CRP and activated C4 or C3, specifically reflects CRP-mediated

complement activation.[6] Moreover, these complexes were detected in several human diseases where CRP presumably is involved in the activation of complement.[6,33–35] We developed a similar ELISA for the quantification of rat CRP–C3 complexes. Control experiments with non-specific polyclonal rabbit IgG and an irrelevant mAb of the same subclass as the anti-C3 mAb, showed the specificity of the ELISA for rCRP–C3 complexes. Using lyso-PC and CPS as ligands, a significant generation of rCRP–C3 complexes was observed in rat plasma. These complexes were not generated during incubation of rat plasma with aggregated IgG, although this complement activator induced significant C4 activation (see Table 1). CRP–C3 complexes were not formed in rat plasma in the presence of high salt concentration, free p-aminophenylphosphorylcholine or EDTA, which are features typical for CRP-dependent complement activation.[6,33,34] Thus, taken together, the results indicated that rat CRP–C3 complexes specifically reflect CRP-mediated activation and are not the result of an innocent bystander phenomenon.

Griselli *et al.*,[10] although postulating that rCRP is unable to activate autologous complement, describe the deposition of rat C3 and rCRP in the ischemic myocardium in a rat acute myocardial infarction model, in agreement with a role for rCRP in the activation of the autologous complement system *in vivo*. Currently, we are investigating complement activation by CRP in various rat models. Our preliminary results show increases in the amount of CRP–C3 complexes in models for hind-limb ischemia reperfusion, liver ischemia reperfusion and bacterial meningitis (data not shown). These findings together suggest that rCRP can also activate autologous complement *in vivo*. The discrepancy between our findings and those of deBeer *et al*., who reported that rCRP is unable to activate complement,[9] is presumably a result of the use of different methods to assess complement activation in plasma. Whereas we used a sensitive assay for a specific activation product, i.e. CRP–C3 complexes, in combination with other assays, deBeer *et al*. used crossed immunoelectrophoresis, which is less sensitive than our ELISAs.

In conclusion, using various approaches and different ligands we show that rat CRP can activate autologous complement. Hence, this property of the protein seems to be widely distributed among animal species. Therefore, we suggest that complement activation upon binding to its ligands is an important function of CRP.

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CHAPTER 3

Estrogen replacement raises rat CRP without enhanced complement activation

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ESTROGEN REPLACEMENT RAISES RAT CRP WITHOUT EVIDENCE OF COMPLEMENT ACTIVATION

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Given current controvøsies regarding anti- and pro-inflammatory effects of estrogen, thae is a need to explore relationships between gonadal hormones and inflammation using appropriate animal models. It has been proposed that rats are not appropriate for such research since, contrary to the effect of estrogen in humans, earlier animal studies had reported that estrogen downregulates serum C-reactive protein (rCRP) levels in the rat. With these considerations in mind, we re-examined the effects of estrogen withdrawal and replacement on CRP expression and complement activation in the rat. F-344 rats und8went bilateral ovariectomy or sham surg8y at 9-10 months of age. Four months lata, ovariectomized rats were treated with traditional high-dose 1 7â-estradiol (Hi-E2) capsules, lowø-dose (Lo-E2) 1 7â-estradiol capsules, or placebo capsules for 7 days prior to sacrifice. Levels of plasma rat C-reactive protein (rCRP) were significantly lower in ovariectomized vs. sham-operated animals (415.5 ± 10.6 vs. 626.6 ± 23.0 mg/L, $p < 0.001$). Estrogen replacement significantly raised rCRP levels in ovañectomized animals (690.0 ± 28.0 mg/L in Lo-E2 and 735.5 ± 35.8 mg/L in Hi-E2, respectively, $p < 0.001$). Plasma rCRP levels correlated significantly with both hepatic rCRP ($r = 0.79$, $p < 0.001$) and serum estradiol ($r = 0.70$, $p < 0.001$) levels. Howeva, no significant differences wae observed in indices of complement activation (C4b/c) or CRP-complement complex genwation (rCRP-C3

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complex). In the mature female rat, ovariectomy reduces and estrogen replacement raises rCRP. Effects of estrogen on plasma rCRP induction are mediated, at least in part, through hepatic mechanisms and do not appear to require or be associated with complement activation.

Keywords Estrogen, Hormone replacement therapy, C-reactive protein, Complement activation, Inflammation

INTRODUCTION

Cardiovascular disease (CVD) remains a leading cause of death in older women (1). Post-menopausal declines in estrogens have been associated with increased production of pro-inflammatory cytokines (2–5). In view of mounting evidence that inflammation may play a critical role in the pathogenesis of atherosclerosis, it has been proposed that declines in estrogen could raise the risk of CVD in this population. C-reactive protein (CRP) is an inflammatory marker and a strong predictor of cardiovascular events in postmenopausal women (6,7). Based on these considerations, hormone replacement therapy (HRT) with estrogen should exert anti-inflammatory effects, yet in most human trials HRT increases CRP levels (8–11). It remains to be seen whether estrogen-mediated elevations in CRP levels may have contributed to the failure of recent randomized trials (12,13) to demonstrate a cardiovascular benefit for HRT.

There is a need for animal studies to explore the relationship between estrogen and CRP, since the mechanisms by which HRT induces CRP remain unknown. It has been proposed that rats are not appropriate for such research since estrogen downregulated serum CRP levels in earlier rat studies (14,15). As an acute response reactant, one of the important host defense functions of human CRP (hCRP) is to induce a self-sustaining inflammatory process through complement activation (16). Although, it had been suggested that rat CRP may be unable to activate complement pathways, Diaz et al. have recently demonstrated the capacity of rat CRP (rCRP) to activate the rat complement system in a manner similar to that of hCRP (17). With the prior considerations in mind, we undertook a study to re-examine the effects of estrogen withdrawal and replacement on CRP expression, as well as complement activation, in the rat.

METHODS

Animal Model

Single colony 240 - 270g female Fisher-344 rats (Harlan Sprague-Dawley) underwent bilateral ovariectomy (OVx) or sham surgery at 9-10 months of age and were maintained in a pathogen-free colony. Four months after

surgery, traditional silastic capsules (Dow Corning; #508-006) containing 100% (about 8 mg per capsule) 17β-estradiol (Hi-E2, Sigma Chem.) were implanted subcutaneously in the dorsal neck for 7 days (18). In other OVx animals, a 1:4 mixture of 17β-estradiol and Cholesterol (Lo-E2)(19) or 100% cholesterol (placebo, Aldrich Chemical Co.) were implanted. The identical animal protocol was repeated in two separate experiments to confirm the findings (n = 5 and n = 6 per treatment group in each of two experiments). Rats were sacrificed following the onset of deep anesthesia using i.p. administration of ketamine (40-60 mg/kg) and xylazine (3-5 mg/kg). Blood samples were collected, kept on ice, and then processed within 3 hr by centrifugation in 1,100 RCF at 4°C for 10 min. Serum or plasma was aliquoted and frozen at −80°C until analysis. All animal care was conducted according to NIH guidelines and all protocols were approved by the Animal Care Committee of the University of Connecticut Health Center.

Serum Estradiol Assay

Estradiol was measured using a 3rd Generation Estradiol RIA kit (Diagnostic Systems Laboratories, Webster, TX, USA) following manufacturer's instructions.

Protein Analysis

Total protein was measured in sera or hepatic homogenates using a BCA protein assay kit (Pierce Chem.) following manufacturer's instructions.

Plasma Rat CRP (rCRP) Analysis

Levels of rCRP were determined by ELISA (17) with a sensitivity of 0.01 mg/ L. The ELISA was performed with a polyclonal rabbit antibody raised against purified plasma-derived rCRP. This antibody does not recognize proteins in rCRP-depleted rat sera and hence is specific.

Hepatic rCRP

Liver tissues were collected, rinsed with ice-cold phosphate buffered saline, pH 7.4 (PBS) x2, and snap frozen at −80°C. Frozen liver tissues were cut to (50 mg per piece and were homogenized in cell-lysis buffer (Cell Signalling Technology) supplemented with protease inhibitor cocktail-I (Calbiochem). Cellular protein was extracted with slow agitation at 4°C for 1 hr and was then centrifuged at 13,000 rpm at 4°C for 20 min. Supernatants were collected and stored at −80°C for rCRP ELISA, as previously described. An aliquot from each sample was taken for protein analysis used to normalize the final rCRP value.

Activated C4 (C4b/c) and rCRP-C3 Complexes

Plasma C4b/c was measured by ELISA as described previously (20). Briefly, the IgG fraction of sheep antiserum against human C4, that crossreacts with rat C4, was used as both a capture, as well as a detection, antibody. Results were expressed as the percentage of the maximal amount of activated C4 generated in rat serum by incubation with purified C1s (Calbiochem). Because the assay does not discriminate between C4b, C4bi, or C4c, the activation product detected was referred to as C4b/c. The ELISA for rCRP-C3 complex quantifies the binding of rCRP to activated C3 (C3b and C3bi) as described by Diaz et al. (17).

Statistics

One-way ANOVA with post hoc tests was used to determine the significance of differences in multiple comparisons. Pearson correlation was used for the relationship of serum rCRP with hepatic rCRP and estrogen. Analyses were performed using SPSS version 11.0. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of Estrogen Deficiency and Replacement on Plasma rCRP Levels

Four months after performing a bilateral OVx in these middle-aged rats, plasma rCRP levels were significantly lower in OVx rats when compared to age-matched sham-operated animals (Fig. 1, 415.5 ± 10.6 vs. 626.6 ± 23.0 mg/ L, $p < 0.001$). Providing E2 replacement to OVx rats significantly increased rCRP levels to 690.0 ± 28.0 mg/ L in Lo-E2 group ($p < 0.001$ vs. placebo-treated OVx animals) and to 735.5 ± 35.8 mg/ L in Hi-E2 group ($p < 0.001$ vs. placebo-treated OVx animals). Ovariectomy resulted in only a small decrease in serum total protein (placebo-treated OVx animals vs. sham: 46.46 ± 2.13 vs. 51.45 ± 4.92 mg/ml, NS). However, as seen with plasma rCRP, E2 replacement induced total protein production in a dose-dependent pattern (57.14 ± 11.49 mg/ml in Lo-E2 group, NS vs. sham; 66.03 ± 14.02 mg/ml in Hi-E2 group, $p < 0.05$ vs. sham, respectively).

Relationships of Peripheral rCRP Levels to Hepatic rCRP and Serum Estradiol

Although CRP synthesis takes place primarily in the liver, CRP can also be expressed in the atherosclerotic arterial wall (21). Because it had been

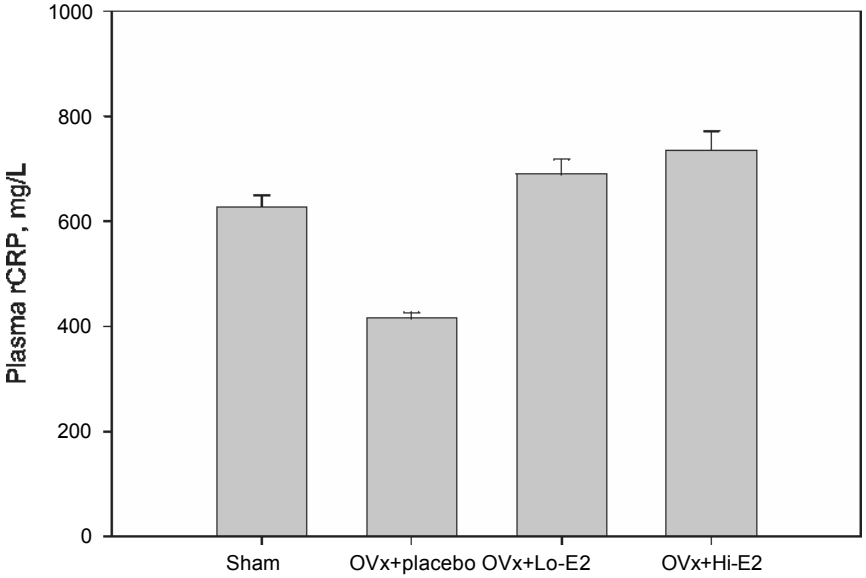

FIGURE 1 Plasma rCRP levels were significantly lower in ovariectomized vs. sham-operated animals (415.5 ± 10.6 vs. 626.6 ± 23.0 mg/ L, n = 11 in OVx+Placebo and n = 10 in sham, *$p < 0.001$). Estrogen replacement in ovariectomized significantly raised rCRP levels vs. ovariectomized receiving placebo (690.0 ± 28.0 mg/ L in Lo-E2 and 735.5 ± 35.8 mg/ L in Hi-E2, respectively, n = 11, $^{\ddagger}p < 0.001$).

proposed that increases in CRP levels following oral HRT merely reflects a first-pass hepatic effect (22), we examined the expression of rCRP in hepatic homogenates and its relationship to plasma rCRP (Fig. 2A). Our data demonstrated that the plasma rCRP levels are significantly correlated with both hepatic rCRP ($r = 0.79$, $p < 0.001$; Fig. 2A) and also serum estradiol ($r = 0.70$, $p < 0.001$; Fig. 2B).

Complement Activation in Relation to Plasma rCRP

To determine the inflammatory consequences and biological significance of E2-mediated rCRP elevation, we examined complement activation by measuring C4b/ c levels, and also by detecting the binding of rCRP to activated C3 (C3b and C3bi), which then generates rCRP-complement complexes (detectable as rCRP-C3 complex). There were no differences among any of the treatment groups (Table 1). In fact, levels of C4b/ c and rCRP-C3 complex were essentially unchanged in animals following OVx with or without E2-replacement. These results indicate that E2-mediated rCRP elevation was not associated with evidence of enhanced complement activation or the generation of rCRPcomplement complexes. Furthermore, neither C4b/ c nor rCRP-C3 complex levels correlated with plasma rCRP or serum estradiol levels (data not shown).

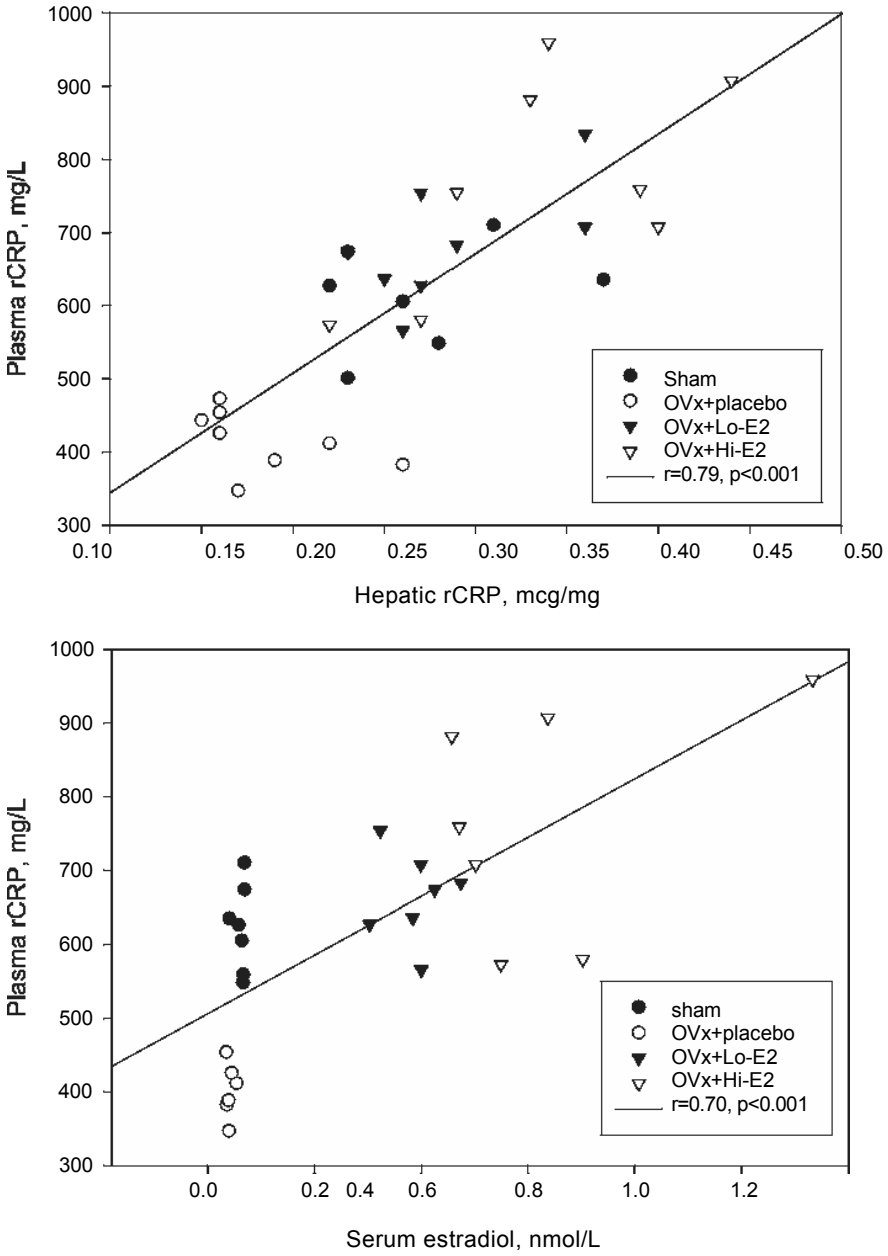

FIGURE 2 The correlation of plasma rCRP with hepatic rCRP and serum estradiol. (A) The levels of plasma rCRP and hepatic rCRP were significantly correlated ($n = 31$, $r = 0.79$, $p < 0.001$). Hepatic rCRP is expressed as mcg of CRP per mg of homogenate protein; (B) Plasma rCRP was also significantly correlated with serum estradiol levels ($n = 27$, $r = 0.70$, $p < 0.001$).

TABLE 1 Levels of plasma C4b/ c and rCRP-C3 complex

	Sham	OVx+Placebo	OVx+Lo-E2	OVx+Hi-E2
C4b/ c, %NRA	13.52 ± 0.90	14.07 ± 0.93	13.11 ± 2.48	10.19 ± 2.44
P value	—	.996*	.980‡	.401‡
95% confidence interval	—	(−5.88□6.98)	(−7.57□5.65)	(−10.49□2.73)
rCRP-C3 complex, U/ ml	0.98 ± 0.05	1.08 ± 0.28	1.04 ± 0.06	0.93 ± 0.17
P value	—	.972*	998‡	.923‡
95% confidence interval	—	(−.55□.75)	(−.69□.61)	(−.83□.53)

C4b/ c levels represent complement activation and are expressed as % of normal rat aged (% NRA), which represents 100% conversion of C4 to C4b/ c (n = 10 for sham and placebo, n = 9 for Lo-E2 and Hi-E2). rCRP-C3 complexes quantify the binding of rCRP to activated C3 (n = 6 for each group, except for n = 5 in Hi-E2). Data are presented as mean ± SEM. The missing values were due to inadequate frozen blood specimens in some animals. There is no significance among groups by One-way ANO VA with post hoc test (* *P* vs. sham, ‡*P* vs. OVx+Placebo).

DISCUSSION

Our study provides the first evidence that, as in humans, 17β-estradiol (E2) replacement increases plasma CRP levels in rats. Our observations are in sharp contrast to previous reports (14,15) which showed that the subcutaneous injection of E2 resulted in decreased serum rCRP concentrations in ovariectomized rats. Although E2 levels were not reported in these studies, the administered doses (1 mg/kg) were most certainly in the pharmacological range (14,15). The silastic capsules used in our study have been shown to release less than 1 μg E2 every 24 hr (23). Thus, subcutaneous injections of 1 mg/kg E2 (14,15) to an adult rat would have delivered a dose of E2 nearly 400 times higher when compared to the doses used in our study. The effects of such extremely high dose E2 on decreasing serum rCRP levels may have been post-translational since it was also reported that hepatic rCRP mRNA levels were unchanged in these animals (15).

Although CRP has been considered to be a sensitive, yet non-specific inflammatory marker, our data indicates that E2-mediated increases in CRP levels do not appear to be associated with evidence of enhanced complement activation. Most earlier studies have primarily focused on the impact of HRT on those pro-inflammatory cytokines (e.g., IL-6) which may mediate CRP production. In contrast, our study examined the important cascade of complement activation in order to evaluate the biological consequences of E2-mediated increases in CRP production. Activation of the complement system via the classical pathway is one of the rigorously defined pathogenic properties of human CRP (hCRP) which might contribute to an active role of CRP in atherogenesis (24,25). However, it had been reported that rat CRP is unable to activate complement (26,27). Given the fact that important biological functions of proteins are probably conserved among species

(25), Hack's group has recently re-evaluated this issue, using various ligands, and tested the capacity of rCRP to activate complement. They have demonstrated that rat CRP, similarly to hCRP, can activate autologous complement upon ligand-binding (17). In fact, CRP is only able to activate complement when it is aggregated to form dimers or trimers. CRP aggregation can be achieved when it is bound to appropriate ligands, such as phosphocholine (20,24,28). Gershov et al. (29) have reported that CRP is able to bind to apoptotic cells, but not to normal cell membranes. Aggregation of CRP upon ligand-binding and the subsequent complement activation generate CRP-complement complexes. Lagrand et al. (30) have reported that CRP colocalized with the deposits of activated C4 and C3 fragments in infarcted human myocardium. Interestingly, Griselli et al. (27) have shown that the administration of hCRP to rats after ligation of the coronary artery enhanced infarct size by 40%. However, *in vivo* complement depletion completely abrogated this effect and markedly reduced infarct size. These findings suggest that complement activation by CRP represents an important mechanism of CRP-mediated tissue damage.

We observed no difference in the levels of generated C4b/ c and rCRP-C3 complexes among sham, OVx/ placebo, OVx/ Lo-E2, and OVx/ Hi-E2 groups. Our results do not provide any evidence of enhanced complement activation following ovariectomy or E2 replacement. An alternative explanation arises from the fact that complement activation by rCRP requires ligand-binding and that rats have unusually high rCRP concentrations under basal conditions; e.g., approximately 300-500 mg/ L (17). With these considerations in mind, it is possible that although rCRP increases upon E2 replacement, this does not result in enhanced complement activation, since levels of appropriate ligands for rCRP do not rise simultaneously and, thus, become limiting. These observations also support our previous reports that C4b/ c and CRP-C3 complex are specific markers for complement activation by human or rat CRP (28,30), and that elevated CRP levels in the absence of appropriate ligands are not associated with enhanced complement activation (28,30). Thus, it is possible that while estrogen-mediated increases in CRP levels may enhance the risk of inflammation, additional conditions (e.g., elevations in levels of CRP ligands) must also be present for complement activation and a full inflammatory response to take place. Our observations indicating no evidence of enhanced complement activation in relation to elevated CRP could help explain several observations from recent clinical trials. In the WHI-OS (8), CRP was an independent predictor of cardiovascular risk for both users and nonusers of hormone therapy. However, use or nonuse of ERT had less importance as a predictor of cardiovascular risk than did CRP levels. Moreover, hormone therapy assignment did not significantly change stratified odds ratios based on CRP levels. Thus, the E2-mediated increase in CRP could not be totally equated with an increased cardiovascular risk.

Although most human studies of oral HRT indicate induction of CRP production, there are considerable controversies regarding both the direction and the magnitude of the effects of HRT on proinflammatory cytokines. Studies evaluating the effect of HRT on circulating IL-6 levels have yielded highly inconsistent results, suggesting that HRT increases (31), decreases (8,32,33), or does not change IL-6 levels (8,34,35). These discrepancies highlight the complex nature of the relationship between estrogen and inflammation. Many types of inflammatory cells are responsive to estrogen (36). Although IL-6 appears to be the major stimulant for hepatic CRP production, mechanisms independent of IL-6 have also been described (37). Other inflammation-associated cytokines, including IL-1â, TNF-á, IFN-ã, and TGF-â may exert additive, inhibitory or synergistic effects on hepatic CRP expression (38). Bruun et al. (39) have reported results of animal studies indicating that ovariectomy significantly increased IL-6 and IL-8 gene expression in rodent adipose tissue, with no apparent effects on TNF-á gene or protein level. Low dose E2 replacement administered at the time of ovariectomy and continued for 5 months prevented these increases. However, no direct effects of E2 on these three adipose tissue-derived cytokines were observed in adipose tissue cultures following 24-hr incubation. These findings suggest that the effect of E2 on these cytokines may be more long-term, or that the *in vivo* effects of E2 on cytokines are mediated indirectly through one or more mediators. It has become apparent that the biological effects of E2 on inflammation are highly complex and clearly not well understood.

Stork et al. (40) reported that combination therapy using 17â-estradiol daily plus standard dose of progestin cyclically had a neutral effect on CRP levels and favorable effects on cell adhesion molecules, suggesting that the type of E2 included in the HRT regimen affects the inflammatory response. Preliminary findings from recent studies suggested that transdermal E2, unlike oral conjugated E2, does not affect CRP adversely (22,34,41,42). Therefore, it has been speculated that the HRT-mediated increase in CRP could be due to a hepatic first-pass effect on CRP production, rather than a true systemic inflammatory reaction. However, we found that E2 also elevates rCRP levels when administered subcutaneously, raising both hepatic and peripheral rCRP levels. Thus, hepatic activation does probably contribute to the increased CRP production following E2 replacement, irrespective of the route of administration. Furthermore, based on our observation of the dose-dependent patterns of rCRP and total serum protein changes in relation to serum E2 levels, we speculate that the increases in levels of rCRP and total protein may both reflect a systemic anabolic effect of estradiol through stimulation of hepatic synthesis. In fact, E2 has been shown to regulate the hepatic production of abundant serum proteins, including albumin (43) and ceruloplasmin (44). In contrast, proinflammatory cytokines, principally IL-6, inhibit the hepatic synthesis of albumin and transferrin in

inflammatory responses (45). Of interest, several recent studies (46–48) have reported that lower oral dose E2 therapy did not raise CRP levels, while conventional doses did. Our data did not show significant differences in rCRP levels between Lo-E2 (25% of estradiol capsule) and Hi-E2 (100% of estradiol capsule) groups. In our study, serum estradiol levels in Lo-E2 rats were still above the proestrus range, yet they were significantly lower than the levels in Hi-E2 animals (Fig. 2B). Nevertheless, we were unable to assess the effects of even lower doses of E2 on inflammation in this rat model.

In conclusion, we found that contrary to earlier reports, ovariectomy significantly reduced and E2 replacement significantly increased plasma rCRP in middle-aged rats. Levels of peripheral rCRP correlated with serum E2 and hepatic rCRP. However, the E2-mediated elevation of rCRP was not associated with enhanced complement activation or rCRP-complement complex formation. Our data support the notion that the E2-mediated increases in CRP may not represent an upregulation of the inflammatory response. Further studies examining yet lower doses of E2 on CRP production and related vascular inflammatory changes are needed.

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CHAPTER 4

C-reactive protein and natural IgM antibodies are activators of complement in a rat model of intestinal ischemia and reperfusion

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Short title: Complement activation by CRP and IgM in intestinal ischemia reperfusion.

Key words: intestine, ischemia, complement, CRP, IgM

Abbreviations: CRP, C-reactive protein; Pc, phosphorylcholine; IgM, immunoglobulin M; I/R, ischemia reperfusion; II/R, intestinal ischemia and reperfusion; Anti-Pc IgM, IgM to phosphorylcholine; C1-Inh, human C1 esterase inhibitor; Alb, human albumin; MPO, Myeloperoxidase.

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Abstract

The role of C-reactive protein (CRP), natural immunoglobulin M (IgM), and natural IgM against phosphorylcholine (anti-Pc IgM) was investigated in relation to complement activation, in a rat model of intestinal ischemia and reperfusion (II/R). The effect of C1-esterase inhibitor (C1-Inh) on this complement activation along with other inflammatory mediators was also studied. Rats were subjected to 1 h of superior mesenteric artery (SMA) occlusion and 3 h of reperfusion. Intravenous administration of vehicle (human albumin) or C1-Inh (200 U/kg) was performed before (n=8) or after ischemia (n=8). II/R increased levels of C4b/c, CRP, IgM, anti-Pc IgM and myeloperoxidase activity in the intestinal homogenates and induced vascular leakage. A good correlation was observed in the intestinal homogenates between C4b/c and CRP levels. Clear depositions of C3, CRP and IgM in intestinal tissue were demonstrated after II/R. In the tissue, a strong correlation of both CRP and IgM with complement was observed. C1-Inh prior to ischemia reduced complement activation response following II/R, as reflected by decreased levels of C4b/c in conjunction with reduced anti-Pc IgM, in the intestinal homogenates. C1-Inh also diminished the leakage of albumin when administrated prior to ischemia. C1-Inh post ischemia reduced C4b/c levels in the homogenates and myeloperoxidase activity. CRP and IgM depositions correlated well with local complement activation, suggesting a role for these molecules in complement activation. Furthermore, C1-Inh potentially inhibited II/R injury either administered prior to or after ischemia, by attenuating complement activation induced by CRP and/or natural antibodies.

Introduction

Induction of ischemia in organs leads to changes in the endothelial cells of the vasculature. These changes include ATP depletion and altered production of bioactive agents, leading to apoptosis and necrosis and finally in loss of organ function. Restoration of blood supply (reperfusion) exacerbates these changes and aggravates endothelial cell damage, at the same time inducing an inflammatory response [1]. II/R injury is associated with a high morbidity and mortality [2]. Additional damage to the injured intestinal tissue is attributed to the activation of the complement system [3-5].

Two activators of the complement classical pathway, i.e. CRP and natural IgM, have been described in relation with ischemia reperfusion (I/R). CRP, the classical acute phase protein in humans, co-localised with activated complement on human infarcted myocardium [6]. In addition, intraperitoneal injection of human CRP increased infarction size in an experimental myocardial infarction model in a complement-dependent fashion in rats [7], which furthermore demonstrated the involvement of CRP in complement-mediated tissue injury following I/R. This co-localisation of CRP and complement may result from cell membranes harbouring binding sites for CRP which can bind to phosphorylcholine (Pc) exposed on the injured membranes, after which the classical pathway is activated [8,9]. In mice, it has been shown that natural IgM participates in the activation of the classical pathway upon II/R [10-12]. A specific self-reactive IgM antibody, able to initiate intestinal and hind limb reperfusion injury in murine models of I/R, was identified [13, 14]. In the infarcted human myocardium IgM colocalized with complement [15]. Krijnen et al., showed that IgM also co-localised with CRP, strongly suggesting that both proteins share a similar specificity, possibly Pc. Others have shown that natural IgM directed against Pc (anti-Pc IgM) binds to damaged cells and plays a role in complement activation [16, 17].

In rat models of II/R, several inhibitors of complement have been evaluated. Administration of the inhibitor prior to ischemia [3, 18] or prior to reperfusion [19, 20] both resulted in a reduction in intestinal injury. C1-Inh is a serine proteinase inhibitor of both the classical and mannan-binding lectin pathway [21]. Administration of human C1-Inh prior to reperfusion attenuated II/R injury in mice [22]. These studies indicate that inhibition of complement activation may be effective in the reduction of I/R induced intestinal injury.

The aim of this study was to investigate the contribution of both CRP and natural IgM in complement activation in an II/R model in the rat, as well as the potential inhibition of this complement activation, using human C1-Inh administration prior to or after ischemia.

Materials and Methods

Animals

Male Wistar rats (Charles River, Broekman Institute B.V., Someren, The Netherlands) weighing 250 to 300 g, were allowed to adjust for one week and were subjected to a regimen of 12:12 h /day-night cycle at constant temperature (22ºC). Food and water were supplied *ad libitum*. Twelve hours before the experiment, animals were fasted with free access to water. All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care and handling of laboratory animals. The Animal Ethics Committee of the Academic Medical Center has approved all experimental procedures.

Intestinal model of ischemia and reperfusion

Animals were weighed and pre-medicated with 4% isoflurane (Florene®, Abbott Laboratories Ltd. Queensborough, Kent, UK) and temgesic (0.033 ml/100 gram bodyweight) (Schering-Plough, Amsterdam, The Netherlands). After endotracheal intubation, the rats were ventilated (Zoovent ventilator, Instruvet, Amerongen, The Netherlands) by inhalation of air: 0_2 (1:1) and 2% isoflurane. Anaesthesia was maintained during the entire experiment. The mean arterial blood pressure was measured via a canula in the carotid artery and was maintained between 80 and 110 mmHg during the entire experiment. Fluid infusion (3 ml per hour with 0.9% NaCl, (Baxter B.V., Utrecht, The Netherlands) was administered via the carotid artery. Body temperature was kept at 37°C and pCO_2 at approximately 35 mmHg.

II/R was induced as previously described [23]. Briefly, using a midline laparotomy, the SMA was clamped with a non-traumatic vascular clip. Clamping was confirmed by immediate blanching of the small intestine and cecum, and lasted for 1 h. Removal of the vascular clip restored blood flow, which was evidenced by the reappearance of the original colour of the intestine. Reperfusion was carried out for 3 h. Sham animals underwent the same surgical intervention except for clamping of the SMA.

Blood samples were collected via the portal vein (1 mL) in siliconized vacutainer tubes at a final concentration of 10 mM EDTA (BD microtainer K2 EDTA tubes, Franklin Lakes, U.S.A), 5 min before ischemia and 10, 60 and 180 min during reperfusion. Plasma was obtained by centrifugation at 1,200 x g for 10 min at 4°C and aliquots were stored at –80°C. Blood loss, due to blood sampling, was corrected by administration of 1 mL of eloHaes (Fresenius Kabi Nederland B.V.,'s Hertogenbosch, The Netherlands). After 3 h of reperfusion the rats were sacrificed by bleeding followed by cardiac-perfusion with 30 mL of 0.01 M phosphate-buffered saline, pH 7.4 (PBS; Fresenius Kabi Nederland) to clear the organs from blood.

A segment of the small intestine was collected 10 cm proximal from the cecum. After weighing the intestinal segments, the lumen was flushed with 30 mL of PBS. The intestine was divided into four sections, which each in turn were divided into four parts. From each section one part was collected (4 parts in total from 4 different sections) and either frozen in liquid nitrogen and stored at –80°C or fixed in 4% (w/v) formaldehyde for further studies. The distal side was marked with a suture.

Blood gas measurement in arterial blood

The values of pH, P_{CO2}, P_{O2}, base excess and HCO_3 were assessed in peripheral arterial blood, collected at the end of the experiment, using the ABL analyzer (Radiometer, Zoetermeer, The Netherlands).

Parameters of intestinal injury

Intestinal injury was assessed using the classification of Park-Chiu [24]. Four different sections of the formaldehyde fixed jejunum from each animal were embedded together in paraffin. Each section was cut at three different levels. 4 μm sections were stained with haematoxylin and eosin. A blinded observer assessed the slides microscopically according to a semi-quantitative scorings methodology. Briefly, the scores used were 0: normal mucosa; 1: subepithelial space at villus tips; 2: extension of subepithelial space with moderate lifting; 3: massive lifting down sides the villi, some denuded tips; 4: denuded villi; dilated capillaries; 5: disintegration of the lamina propia; 6: crypt layer injury; 7: transmucosal infarction; and 8: transmural infarction.

Myeloperoxidase activity
Myeloperoxidase (MPO) activity, an index of neutrophil infiltration, was measured in the intestine as described by Krawisz *et al*. [25] with minor modifications. Briefly, 30-50 mg of intestinal tissue was homogenised (Heidolph, Diax 900) in 5 mM sodium phosphate buffer, pH 6.0. After a sample was taken from the homogenate for protein content determination (Pierce Biotechnology, Inc Rockford IL, USA), it was centrifuged for 10 min at 12,000 x g at 4ºC. The pellet was homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich Chemicals BV, Zwijndrecht, The Netherlands) and 10 mM EDTA in 50 mM sodium phosphate buffer, pH 6.0. Homogenates were freeze-thawed three times, pottered 10 times (Bellco 20, RW 20.n IKA Labortechnik), sonicated for 10 sec and centrifuged at 12,000 x g at 4ºC for 10 min. The supernatants were collected and assayed for MPO activity by addition of 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma-Aldrich Chemicals) and 0.001% H_2O_2 (Sigma-Aldrich Chemicals) in 50 mM sodium phosphate buffer, pH 6.0. The change in absorbance was measured spectrophotometrically (Victor^2, Wallec 1420) at 450 nm during 10 min at 37ºC. One Unit is defined as the amount of enzyme necessary to produce a change in absorbance of 1.0 per min. The MPO activity was expressed as units per mg protein.

C1 esterase inhibitor and albumin administration
Human C1-Inh (200 U/kg bodyweight) (Cetor, Sanquin Research, Amsterdam, The Netherlands) was administered i.v. via the penile vein either 5 min before ischemia (C1-Inh+ II/R) or 5 min before reperfusion (II/R+ C1Inh), (n=8 per group). Human albumin (Alb) (Cealb, Sanquin Research), administrated in a similar fashion and in an equal protein load as C1-Inh, served as a reference for the intervention with C1-Inh (Alb+ II/R and II/R+ Alb, respectively, (n=8 per group). Furthermore, four groups (n=6 rats per group) were included to investigate the effects of C1-Inh or Alb in the anaesthetised rats (sham groups). One hour of ischemia was replaced by one hour of anaesthesia. In total, four randomised groups were used to investigate the effects of C1-Inh administration prior to ischemia. Four additional randomised groups were used to investigate the effects of C1-Inh after ischemia.

Preparation of the intestinal homogenates
Intestinal tissue (1 part of the frozen sections, as described above) was crushed upon freezing in liquid nitrogen. All powders were stored at –80°C until use. All following procedures were performed at 4°C. Pulverised tissue was resuspended in veronal buffered saline (0.1%, w/v, Tween 20, 0.5 M NaCl, 10 mM EDTA, pH 7.4, final concentrations) and incubated for 4 h. This suspension was centrifuged (12,000 x g for 20 min). The supernatant was used for further analysis.

Determination of total protein and albumin concentration
Protein content in both plasma and in the intestinal homogenates was measured using the BCA protein assay (Pierce Biotechnology, Inc Rockford IL, USA). Albumin was quantified in the intestinal homogenates using the rat albumin ELISA quantitation kit (Bethyl laboratories, INC, West FM 1097 Montgomery, TX, USA).

ELISA for C4b*/c and CRP quantification*
The levels of activated C4 were used as parameter for complement activation. They were measured in plasma and in intestinal homogenates as described previously [26]. As the assay does not discriminate between C4b, C4bi or C4c, the activation product detected was referred to as C4b/c. Results were expressed as percentage (%) of C4b/c in the samples compared to

that of aged normal rat plasma (NRA). NRA was used as a house standard in this ELISA. Rat CRP was quantified by ELISA as previously described [27]. Results were expressed as μg/mL**.** The C4b/c and CRP levels measured were adjusted to the total protein concentration in each sample.

ELISA for C1-Inh, IgM and anti-Pc IgM quantification

The levels of human C1-Inh, natural rat IgM and anti-Pc IgM were measured both in plasma and in the intestinal homogenates. C1-Inh was detected as described before [26, 28]. Results were quantified by comparison with a standard consisting of normal rat plasma supplemented with 250μg/mL (equivalent to 1 U/ ml) of human C1-Inh.

IgM and anti-Pc IgM were quantified as follows. Maxisorp plates (Nunc Band Products, Denmark) were coated with goat anti-rat IgM (μ chain specific), (SouthernBiotech, Inc, Birmingham, USA) at 1 μg/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.6 overnight at 4°C for the quantification of IgM. Instead of goat anti-rat IgM, the plates were coated with Pc-BSA (Biosearch Technologies, Inc. 81 Digital Drive Novato CA, USA) for anti-Pc IgM quantification. Final volume in the wells was 100 μl, unless stated otherwise. After washing the plates twice with PBS, residual binding sites were blocked with BSA (Intergen company, Purchase, NY , USA) at 2% (w/v) in PBS (200 μl/well) for 1 h at room temperature. The plates were then washed with PBS/0.02% (w/v) Tween 20. Samples to be tested were diluted in veronal buffered saline containing 10 mM EDTA (to prevent calcium-dependent CRP binding to Pc), 0.1% (w/v), BSA, 0.1% (w/v) Tween 20, pH 7.4, (assay buffer). Bound rat IgM was detected by goat anti-rat IgM labelled with HRP (Bethyl laboratories, INC, West FM, and Montgomery, USA) diluted in assay buffer. Finally, plates were washed and peroxidase activity was visualized by incubation with 3,3', 5,5',-tetra-methyl-benzidine, 100 μg/ml in 0.11 M sodium acetate, pH 5.5, containing 0.003% (v/v) H_20_2. The reaction was stopped after 10 min by addition of 2 M H_2S0_4, and the absorbance at 450 nm was measured with a Titertek plate reader. C1-Inh, IgM and anti-Pc IgM levels were also adjusted to the total protein concentration in each sample.

Immunohistochemistry

Depositions of activated rat C3, CRP and IgM were assessed using frozen sections (4 μm thick) from the proximal and the distal part of the jejunum (two sections instead of the four combined for the homogenates). Rabbit polyclonal antibodies against rat C3 (gift from Dr. Daha M. R., Leiden University Medical Center, Leiden, The Netherlands) and CRP [27], followed by the polyclonal goat anti-rabbit immunoglobulins/HRP (DAKO Cytomation, Glostrup, Denmark) were used to detect C3 and CRP deposition. Deposition of rat IgM was examined with the goat anti-rat IgM labelled with HRP (Bethyl laboratories, INC, West FM, and Montgomery, USA). Irrelevant rabbit IgG and goat IgG were used as isotype controls. 3, 3'-diaminobenzidine-tetrahydrochloride (Sigma, St Louis, Missouri, USA) at 0.5 mg per ml in PBS, pH 7.4, containing 0.01% (v/v) H_2O_2, was used as substrate. The slides were counterstained with haematoxylin for one min, dehydrated, cleared, and finally mounted. The extent of depositions was examined both in the mucosa and in the mucosal debris present in the lumen and was quantified using a video overlay system (QPRODIT 5.2, Leica) [29]. Each tissue slide was subdivided into five parts at magnification 250 times. For the mucosa, a photo of each part was taken and the total (cm^2) and the positive area (cm^2) for C3, CRP, and IgM was calculated and expressed as the percentage of deposition. Finally, the mean value of the percentage determined from the five parts was used for analysis. The percentage of the microscopic field positive for C3, IgM and CRP was also assessed for mucosal debris present

in the lumen of each slide. Two investigators judged and scored all slides without knowledge of the experimental groups. The final scores were obtained after consensus between the two investigators. No staining for C3, CRP or IgM was observed with irrelevant rabbit or goat polyclonal antibodies.

Data analysis

Data were expressed as mean ± SEM in text, tables, and figures except for the Park Chiu score for which median values were used. Statistical analysis was assessed by Kruskal Wallis / Mann-Whitney using Statistical package for the Social Sciences (SPSS 11.5, SPSS Inc, Chicago, Illinois, United States of America). Correlations between parameters were assessed by estimating Spearman's rank correlation coefficient (Rs). Plasma parameters measured were evaluated by ANOVA for repeated measurements. $p<0.05$ is considered significantly different.

Results

All animals (n = 56) in the experiment were included. The average body weight of the animals was 280 ± 1.3 gram. The pH, P_{CO2}, P_{O2}, HCO_3 and base excess in the arterial blood at the end of reperfusion were indicative of proper ventilation strategies during the entire experiment. After administration of C1-Inh, its plasma level was significantly increased, as expected. Differences between the ischemic or sham groups were not observed. In addition, plasma levels of C4b/c, CRP, IgM and anti-Pc IgM adjusted for total plasma protein concentration did not differ between the sham and II/R groups (data not shown).

Intestinal injury and neutrophil sequestration

As expected, intestinal injury following I/R was significantly increased compared to sham operated animals, as reflected histologically by complete disintegration of the lamina propia (5.0 vs. 0.5 in sham and II/R, respectively, $p <0.05$) Figure 1A). Increased levels of MPO activity were measured in homogenates from animals receiving albumin after 1 h of ischemia and 3 h of reperfusion compared to the sham operated animals (Figure 1B). Although inhibition of the classical and mannan binding lectin pathway of the complement system by C1-Inh did not show a significant reduction of morphological injury (Figure 1A), it reduced the MPO levels significantly when administered immediately before the reperfusion period (Figure 1B).

Complement activation and CRP, IgM and anti-Pc IgM levels in the intestinal homogenates

Complement activation following II/R, as measured by C4b/c levels, was significantly increased (Figure 2A). In association with complement activation, levels of CRP, IgM and anti-Pc IgM were also increased (Figure 2B-D). The increased levels of C4b/c after II/R injury were reduced after administration of C1-Inh either before or at the end of ischemia, although these did not reach the level of the sham group. C1-Inh administered before ischemia not only reduced complement activation but also significantly decreased the anti-Pc IgM level (Figure 2D). However, no effect of C1-Inh on CRP and IgM levels was detected in the intestinal tissues after I/R (Figure 2 B-C).

To assess the relationship between CRP, IgM, anti-Pc IgM and their contribution to complement activation, the correlation of these parameters using Spearman's rank correlation test was analysed (Table 1). A good correlation between complement activation (C4b/c) and

increased CRP levels (Rs 0.769, $p<0.01$) was observed. C4b/c and IgM levels were not significantly correlated, nor were the C4b/c and anti-Pc IgM levels. There was correlation however between CRP versus IgM and CRP versus anti-Pc IgM.

Vascular leakage

II/R increased intestinal wet weight- versus bodyweight ratio ($p<0.05$). C1-Inh did not reduce the intestinal wet weight -versus bodyweight ratio. The effect of vascular permeability after I/R was evaluated by measurement of the albumin content in the intestinal homogenates. The groups with II/R had increased levels of albumin compared to these of the sham groups ($p<0.05$). A significant reduction was observed after C1-Inh administration prior to ischemia (data not shown).

Deposition of complement, CRP and IgM in intestinal tissue after I/R.

Immunohistochemical assessment of intestinal frozen sections was performed to confirm that the observed increase in complement activation, CRP and IgM in the intestinal homogenates, was a result of actual binding to the membrane and not due to vascular leakage. In the sham-operated rats, no depositions of C3 (A), CRP (B), or IgM (C) were detected (Figure 3). C3 depositions were observed on the intestinal mucosa, in the subepithelial space, as well as on (damaged) mucosal fragments within the lumen of the intestine after 1 h of ischemia and 3 h of reperfusion (D). CRP and IgM were also deposited upon II/R (E and F, respectively). The percentages of C3, CRP, and IgM depositions through out the mucosa and the lumen were significantly increased in the II/R groups compared to the sham groups (Table 2 A, B).

Macroscopic examination showed marked differences in appearance of the ischemic segments of the intestine after I/R, with more evidence of infarction, edema, and haemorrhage in the distal segments. It was associated with a significant increase in the areas of the mucosa in the distal part compared to the proximal parts of the intestines (90.7 cm^2 ± 7.3 vs. 69.6 cm^2 ± 5.1). In order to see whether these macroscopic differences were related to increased exposition of binding sites for CRP and/or IgM and increased complement activation in the distal intestinal segments, the percentages of depositions for all proteins in the proximal versus the distal frozen sections were compared (Wilcoxon matched pairs test, results not shown). Indeed, both CRP and C3 depositions were significantly increased in the distal segments of the mucosa.

Administration of C1-Inh before reperfusion resulted in reduction of the percentage of C3 depositions in the lumen compared to the rats given albumin before reperfusion (73.1 ± 6.0 vs. 48.5 ± 7.0, for II/R+ Alb and II/R+C1-Inh respectively). This reduction was however not significant (p 0.091). Other effects of C1-Inh on the depositions of C3, CRP, and IgM were not detected.

Correlation between C3, CRP, and IgM depositions

To study the relative contribution of CRP and IgM to complement activation, correlations were performed between percentages of C3, CRP and IgM deposition in the lumen and in the mucosa. Strong correlations were observed between the percentages of depositions of C3-CRP, C3-IgM, and CRP-IgM in the distal part of the lumen and for C3-CRP and CRP-IgM in the proximal part of the lumen. No correlation between C3-IgM in the proximal part of the lumen was found (Table 3). There was no correlation between the percentages of depositions of CRP and IgM, and complement in the proximal part of the mucosa. In addition, a weaker correlation between C3-CRP and CRP-IgM was found in the distal part of the mucosa in comparison to the proximal part (Table 3).

Discussion

A role of the complement system in I/R injury in various organs including the intestine is well documented. However, the exact mechanisms triggering complement activation in II/R, is not completely clear. In mice, a role of natural IgM antibodies herein has been postulated [10-12]. In humans, both IgM and CRP seem to play a role in complement activation after cardiac I/R injury [15]. In the present study, 1 h of intestinal ischemia followed by 3 h of reperfusion led to depositions of C3, CRP and IgM in intestinal tissue. This was associated with increased levels of complement, CRP, IgM, anti-Pc IgM in intestinal homogenates from rats subjected to II/R. C1-Inh administration either prior to or after ischemia inhibited the classical pathway of complement activation as observed in the homogenates. However, the decrease in complement deposition found upon C1-Inh treatment was not significant. Protective effects of C1-Inh decreasing vascular leakage and neutrophil infiltration in the ischemic intestinal tissue were also observed when it was administered before or after reperfusion respectively.

Endogenous CRP was found to localize in ischemic myocardium in a rat model of acute myocardial infarction [7]. The present study demonstrated that post ischemically reperfused intestinal tissue not only contained CRP but also IgM, implying that at least two different proteins may also be involved in II/R-induced complement activation. The correlation between CRP and C4b/c deposition was better than this between IgM and C4b/c levels in the homogenates. These results suggest that CRP is more important for complement activation in this model than IgM. However, in the mucosal debris in the lumen a good correlation between C3 and both CRP and IgM was observed (Rs 0.698 and Rs 0.637 respectively). It is possible that in animals with low CRP and normal IgM levels (such as mice); IgM will be the dominant inducer of complement activation during I/R injury. In animals with high CRP levels and normal IgM levels such as the rat, both CRP and IgM are likely to be involved in complement activation.

The reason for the observed increase in depositions of complement activators in the distal part of the lumen compared to the proximal part is not clear. The increased binding of C3, CRP and IgM in the distal part of the intestine cannot be explained by collaterals, since shunting via collateral blood flow would have resulted in less tissue damage. Rinsing of the intestine after excision may be a more likely explanation, as this will result in an increase in luminal mucosal debris more distally. There seems to be a high degree of similarity between the correlations for the various components observed in the homogenates in comparison to the depositions found in the lumen of the intestine. Some difference in correlation coefficients between the homogenates and the depositions may be because the homogenates represent the mean of the combined lumen and mucosa (distal and proximal). Apparently, injured mucosa is able to bind complement activators that will be lost in the lumen. Less injured mucosa will remain attached. Consequently, less complement activators will bind to the intact mucosa.

Complement activation as measured in the intestinal homogenates was inhibited by C1-Inh administration either prior to or after ischemia. The levels of anti-Pc IgM in these homogenates were significantly reduced by C1-Inh administration prior to ischemia, in contrast to CRP and IgM levels. C1-Inh may prevent membrane changes owing to decreased insertion of the membrane attack complex in the membrane. A reduction in membrane changes leads to reduced exposure of Pc epitopes, resulting in reduced binding of anti Pc-IgM. However, both anti-Pc IgM and CRP are able to bind to Pc in ischemic tissue as measured in the homogenates. CRP can apparently still bind to the exposed Pc, while anti-Pc

IgM shows reduced binding upon C1-Inh administration. The different effect of C1-Inh on anti-Pc IgM and CRP binding to the ischemic intestinal tissue also suggests that anti-

Pc IgM and CRP have different binding affinities for Pc. Recently, two types of membrane-associated neoantigens for Pc were detected on apoptotic cells and on oxidized low density lipoprotein [30]. This suggests subtle variations in the exposure of specific Pc epitopes, which may reflect the stage of apoptotic cell death or the biological context of the tissue that expresses Pc as haptens. This could also be true for CRP and anti-Pc IgM binding to Pc epitopes in ischemic intestinal tissue. Another mechanism to explain the inhibitory effect of C1-Inh on the reduction of anti-Pc IgM levels could be that both molecules bind to the same structure on the damaged intestinal cell membranes. However, the binding of C1-Inh to Pc molecules has not been identified. Moreover, the exact antigen recognized by IgM antibodies has not been identified. It has been reported that another ligand than Pc is involved as well in the binding of natural IgM to ischemic cells such as a protein of about 250 KDa presenting on the damaged intestinal tissue [13]. Analysis of this high molecular mass band yielded peptide sequences homologous to nonmuscle myosin heavy chain type II C. A synthetic peptide representing a conserved region of nonmuscle myosin heavy chain type II C significantly blocked intestinal reperfusion injury mediated by IgM in immune deficient mice [31]. It is known that IgM binding to ischemic skeletal muscle already starts at the onset of ischemia and proceeds during reperfusion [32]. Therefore, inhibition of IgM binding by C1-Inh could be an additional protective action of this drug.

C1-Inh reduces neutrophil influx as measured by MPO levels and vascular leakage. This protective action of C1-Inh has been reported and may be a direct effect of C1-Inh binding to selectins expressed on the ischemic endothelial cell membrane [33]. Hence, via this effect C1-Inh may prevent extravasation of neutrophils, resulting in reduced formation of pro-inflammatory substances. However, an alternative mechanism to explain the effect of C1-Inh on neutrophil influxes may be the reduction of C5a formation, a potent anaphylatoxin, via a reduction in complement activation. C1-Inh (200 U/kg bodyweight) administered in a mouse model of 30 min of intestinal ischemia and 2 h of reperfusion resulted in beneficial effects on the mucosa [22]. In this study however, no beneficial effect on the mucosa was observed. A higher dose might have been more effective, since C4b/c activation was not completely reduced by C1-Inh. Another reason may be the severity of the intestinal damage induced upon 1 h of ischemia and 3 h of reperfusion. The damage inflicted to the intestine may have been too severe and therefore less responsive to treatment.

In conclusion, the observed strong correlation of both CRP and IgM in relation to complement activation in intestinal ischemic tissue is suggestive that both compounds contribute to this activation process. C1-Inh inhibits complement activation when administered either prior to or after the ischemic period. C1-Inh potentially inhibits II/R induced injury either administered prior to or after ischemia.

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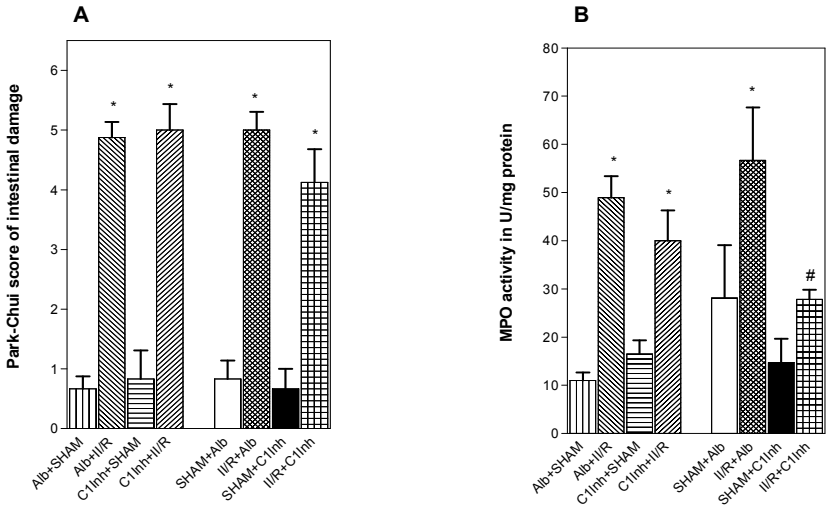

Figure 1. Effect of C1-Inh on ischemia reperfusion induced mucosal injury and neutrophil infiltration (MPO) after ischemia and reperfusion. Rats were injected i.v. with Alb or C1-Inh either prior (Alb+II/R; C1-Inh+II/R) to or after (II/R+Alb; II/R+C1-Inh) 1 h of ischemia. After 3 h of reperfusion, mucosal injury (**A**) and MPO activity were determined (**B**). Each bar represents the median (A) or mean ± SEM (B) with 6-8 rats per group. ***** Significant compared to SHAM group, $p<0.05$; # significant compared to the II/R+Alb group, $p<0.05$ (by Kruskall-Wallis/Mann-Whitney). A p-value <0.05 was considered significant. C1-Inh did not reduce intestinal mucosal injury. However, MPO activity indicating neutrophil infiltration in the intestinal tissue was reduced when C1-Inh was administrated after ischemia.

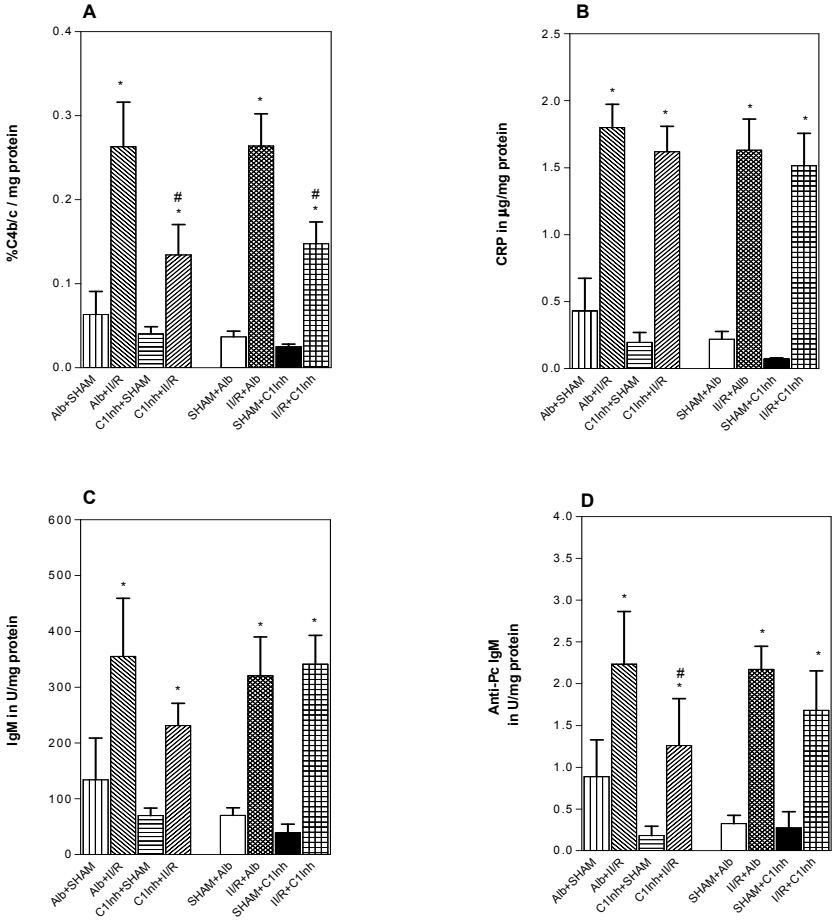

Figure 2. C4b/c, CRP, IgM and anti-Pc IgM levels in the intestinal tissue after I/R and effect of C1-Inh. C4b/c (**A**), CRP (**B**), IgM (**C**) and anti-Pc IgM (**D**) levels in the homogenates were measured and corrected for total protein concentration. Each bar represents the mean ± SEM with 6-8 rats per group. * Significant from the SHAM group, $p<0.05$; # significant from the II/R+Alb group, $p<0.05$ (by Kruskall-Wallis/Mann-Whitney). Complement activation (A) and increased levels of CRP (B), IgM (C) and anti-Pc IgM (D) were found after II/R. C1-Inh administration prior to or after ischemia reduced complement activation but did not affect the CRP and IgM levels. C1-Inh administration prior to ischemia reduced anti Pc-IgM levels.

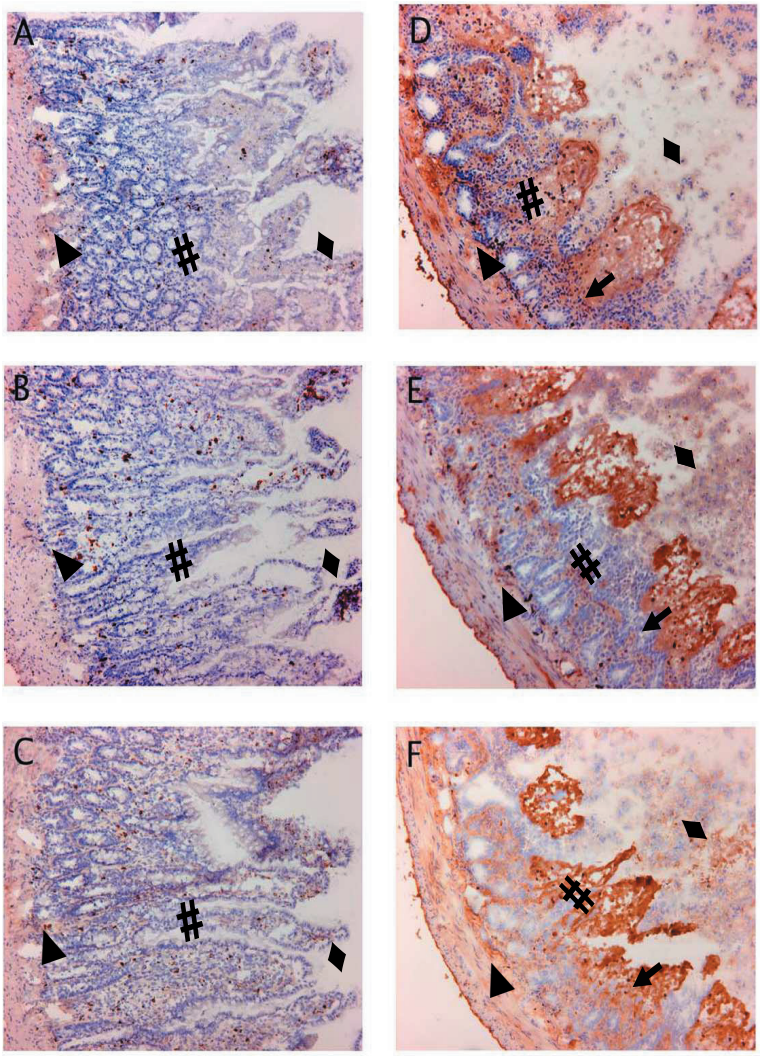

Figure 3. Immunohistochemical assessment of C3 (A, D), CRP (B, E) and IgM (C, F) in a series of sections of normal intestines (A, B, C) and intestines subjected to one hour of ischemia and 3 h of reperfusion (D, E, F). In the subepithelial space (▲), the intestinal mucosa (#) and fragments of tissue in the intestinal lumen (♦), clear staining (*arrowheads*) is shown for C3, CRP and IgM in the rats subjected to intestinal ischemia and reperfusion. Rats of the control group show no specific staining. The sections shown are representative for that particular experimental group (magnification x 250).

Variable 1	Variable 2	Rs*	p-value
C4b/c	CRP	0.769	<0.01
C4b/c	IgM	0.406	0.133
C4b/c	anti-Pc IgM	0.334	0.224
CRP	IgM	0.529	0.043
CRP	anti-Pc IgM	0.511	0.051

*Rs: Spearman‘s coefficient of correlation

Table 1. Correlations between the levels of C4b/c, CRP, IgM and anti-Pc IgM present in the intestinal homogenates after intestinal ischemia and reperfusion injury. Levels in the homogenates were normalized for protein content. Spearman’s rank coefficient of correlation (Rs) was used to determine relation between mediators. A p-value < 0.05 was considered significant. Increased CRP levels correlated well with complement activation.

A

	Sham (mean, SEM)		I/R (mean, SEM)		
	proximal	distal	proximal	distal	p-value
C3	0	0	5.1 (0.9)	24.4 (6.8)	0.0001
CRP	0	0	13.7 (2.9)	34.1 (7.1)	0.0001
IgM	0	0	27.5 (3.7)	40.1 (6.2)	0.0001

Mann-Whitney test two tailed, to compare the percentages of depositions for C3, CRP and IgM in the ischemic vs. control group.

B

	Sham (mean, SEM)		I/R (mean, SEM)		
	proximal	distal	proximal	distal	p-value
C3	0.8 (0.5)	1.2 (0.6)	58.8 (8.0)	81.0 (7.2)	0.0001
CRP	0	0	78.3 (6.5)	90.0 (3.9)	0.0001
IgM	4.1(0. 6)	7.5 (3.7)	84.0 (4.2)	93.3 (2.1)	0.0001

Mann-Whitney test two tailed to compare the percentages of depositions for C3, CRP and IgM in the ischemic vs. control group.

Table 2. Semiquantitative analysis of C3, CRP and IgM deposition in the mucosa (A) and in the lumen (B) of the intestines. Extent of the deposition on the proximal and distal part of the intestine is expressed as the mean ± SEM of the percentages of positive areas for each protein as described in *Material and Methods*. Two tailed Mann-Whitney test was used, to compare the percentages of depositions for C3, CRP and IgM in the ischemic vs. control groups. *A p-value <0.05 was considered significant. Complement, CRP and IgM are deposited in the mucosa and in the lumen of the jejunum only in the rats subjected to 1h of ischemia and 3 h of reperfusion.

A

Proximal part		Rs	p-value	n
Variable 1	Variable 2			
C3	CRP	-0.005	0.859	16
C3	IgM	0.051	0.850	16
CRP	IgM	0.149	0.579	16
Distal part				
Variable 1	Variable 2			
C3	CRP	0.508	0.044	16
C3	IgM	0.380	0.145	16
CRP	IgM	0.545	0.029	16

B

Proximal part		Rs	p-value	n
Variable 1	Variable 2			
C3	CRP	0.750	0.002	14
C3	IgM	0.355	0.210	14
CRP	IgM	0.656	0.008	15
Distal part				
Variable 1	Variable 2			
C3	CRP	0.698	0.005	14
C3	IgM	0.637	0.011	15
CRP	IgM	0.863	0.0001	15

Table 3. Correlations between C3-CRP, C3-IgM and CRP-IgM depositions on intestinal ischemic tissue (both II/R groups with Alb). Correlations were calculated using the Spearman‘s correlation coefficient (Rs). Sections from the proximal and distal part of the intestines were analyzed in the mucosa (**A)** and in the lumen **(B).** A p-value < 0.05 was considered significant. Ns: not significant. The correlation between C3-CRP, C3-IgM and CRP-IgM was stronger in the distal part of the lumen compared to the proximal part of the lumen and, compared to both mucosal parts.

CHAPTER 5

Levels of natural IgM antibodies against phosphorylcholine in healthy individuals and in patients undergoing isolated limb perfusion

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Levels of natural IgM antibodies against phosphorylcholine in healthy individuals and in patients undergoing isolated limb perfusion

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Abstract

Natural IgM antibodies against phosphorylcholine (anti-Pc IgM) resemble C-reactive protein (CRP) regarding specificity and have gained increasing attention because of their supposed role in clearance of damaged cells and in cardiovascular disease. In order to quantify these antibodies in human plasma, we have developed an ELISA system, in which *p*-aminophenylphosphorylcholine (PCH) coupled to human serum albumin (HSA) was coated on microtiters plates. Human plasma or serum samples were incubated in the plates, after which bound anti-Pc IgM was detected with mouse anti-human IgM-HRP. Pre-incubation of Plasma with competitors such as phosphorylcholine, phosphorylethanolamine, phosphorylserine or glycine–HSA, confirmed that the ELISA was specific for anti–Pc IgM. Levels of anti-Pc IgM in a cohort of healthy donors differed by more than 100-fold, whereas the fluctuation of anti-Pc IgM levels in individuals over time was small (coefficient of variation between 6% to 25%). Furthermore, there was no correlation between CRP and anti-Pc IgM in this cohort. Levels of anti-Pc IgM in the normal donors correlated significantly with IgM binding to apoptotic cells. To test the hypothesis that anti-Pc IgM can bind to neo-antigens expressed on necrotic or apoptotic cells, anti-Pc IgM was also quantified in patients with tumors undergoing isolated limb perfusion with tumor necrosis factor-α (TNF-α). Following this procedure, a significant decrease of circulating anti-Pc IgM relative to total IgM was found in all five patients tested.

Abbreviations: abs, antibodies; anti-Pc IgM, anti-phosphorylcholine IgM antibodies; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride); HSA, human serum albumin; mAb, monoclonal antibody; NHP, normal human plasma; o/n, overnight; oxLDL, oxidized low density lipoprotein; PBS, phosphate buffered saline, pH 7.4; Pc, calcium salt of phosphorylcholine; PCh, *p*-aminophenylphosphorylcholine; PE, *O*-phosphoryl-ethanolamine; PS, *O*-phospho-L-serine; rhCRP, recombinant human C-reactive protein; RT, room temperature; strept-PO, streptavidin-coupled to peroxidase; TNF-α, tumor necrosis factor-α; VB, veronal-buffered saline containing 2 mM $CaCl_2$, 10 mM $MgCl_2$, pH 7.4.

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In conclusion, we have developed a specific and reproducible ELISA for anti Pc IgM quantification. Fluctuation of levels of these natural antibodies over time in healthy individuals was limited, although the variation among individuals was large. Significant decreases of levels of anti-Pc IgM were found to occur during tissue damage.

Keywords: Natural antibodies; Inflammation; ELISA; C-reactive protein

1. Introduction

Antibodies against phosphorylcholine (anti-Pc abs) have been studied for a few decades and are thought to constitute a first line defense against infections by virulent *Streptococcus pneumonia* and possibly other bacteria (Briles et al., 1981; Mold et al., 2002). These antibodies (abs) belong to the class of natural antibodies (Kantor et al., 1997), and in mice frequently carry the so-called T15 idiotype, which initially was identified in an IgA paraprotein secreted by the plasmacytoma line T (EPC) 15 (Sigal et al., 1975; Gearhart et al., 1975). More recently, high titers of anti-Pc abs have been described in hypercholesterolemic apolipoprotein E-deficient mice that develop severe atherosclerotic disease (Palinski et al., 1996). Most of these anti-Pc abs are of the IgM class, and are produced by CD^{5+} B1-cells, the predominant B lymphocytes in the newborn. Further characterization of these abs revealed that they are structurally and genetically related, if not identical, to the classic T15 idiotype carrying abs (Kono et al., 1993). Both T15 anti-Pc abs as well as the abs occurring in atherosclerotic mice recognize the Pc head group moiety of phosphatidylcholine in oxidized low density lipoprotein particles (OxLDL) (Shaw et al., 2000; Kearney, 2000; Palinski et al., 1996; Friedman et al., 2002). Both types of abs also bind to oxidized Pc-containing phospholipids on apoptotic cells but only poorly, if at all, to native LDL or non-oxidized Pc-containing phospholipids (Kim et al., 2002).(Chang et al., 1999; Shaw et al., 2000, 2003; Friedman et al., 2002; Palinski et al., 1996).

Anti-Pc abs presumably play a role in the pathogenesis of atherosclerosis although their precise role is not known (Rose and Afanasyeva, 2003). Among their supposed functions is blockade of uptake of OxLDL by macrophages (Horkko et al., 1999). Accordingly, anti-Pc abs may slow down progression of atherosclerosis by inhibiting the transformation of macrophages into foam cells. Recently, Binder et al. (2003) have shown that circulating levels of natural anti-Pc IgM in LDL receptor-deficient mice increase upon vaccination with *S. pneumoniae.* Importantly, this immunization attenuated progression of atherosclerosis, suggesting a protective effect of anti-Pc IgM in this murine model.

Based on their supposed role in murine models of atherosclerosis, studies on the relationship between circulating levels of anti-Pc IgM and the risk of cardiovascular disease are warranted. To this end, specific and reproducible assays for these abs are needed. In the present study, we developed a specific and reproducible ELISA for the quantification of anti-Pc IgM in humans. Using this assay, the distribution of anti-Pc IgM in a cohort of healthy donors was estimated. In addition, the correlation between plasma levels of anti-Pc IgM and those of IgM binding to apoptotic cells was assessed. Finally, levels of this IgM were studied in five patients with malignant tumors undergoing isolated limb perfusion with tumor necrosis factor-α (TNF-α), to assess whether levels of anti-Pc IgM decrease in situations of enhanced tissue destruction.

2. Materials and methods

2.1. Patients and healthy individuals

For the present study, the following sets of plasma samples were tested: (1) samples from healthy lab donors obtained at 1-week interval during a period of 6 weeks; (2) samples from healthy blood donors; (3) serial samples from five patients with a limb tumor (sarcoma or melanoma) who were treated with isolated limb perfusion with TNF α (Boehringer Ingelheim; 4 mg/leg or 3 mg/arm). These patients participated in studies on the effect of isolated limb perfusion on local and systemic activation of coagu-

lation and inflammatory systems (Vrouenraets et al., 1999). TNF-α was administered during a 3.5-h limb perfusion. Blood samples were drawn before and at several times points after the perfusion up to 48 h. Baseline samples and samples obtained at 24 or 48 h after the perfusion were used for the present study.

2.2. Collection of blood samples

The blood samples were collected in siliconized vacutainer tubes containing sodium citrate, EDTA or sodium heparin (Greiner, Kremsmunster Bad Haller, Austria). Plasma was obtained after centrifugation at $1300 \times g$ for 10 min at 4 °C. To obtain recalcified plasma, sodium citrate or EDTA plasma was recalcified by incubation with 12 mM $CaCl_2$ for 15 min at 37 °C. The formed fibrin clot was removed by centrifugation as described above. Fresh normal human serum was obtained by collection of blood in glass tubes. Blood was allowed to clot for 1 h at room temperature (RT), and centrifuged as described above. Serum and plasma samples were stored in aliquots at −70 °C.

2.3. Reagents, proteins and antibodies

p-Aminophenylphosphorylcholine (PCh), the calcium salt of phosphorylcholine (Pc), was obtained from Sigma (St Louis, MO, USA). *O*-phosphorylethanolamine (PE) and *O*-phospho-L-serine (PS) were obtained from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as well as gel consisting of immobilized *p*-aminophenylphosphorylcholine were obtained from Pierce Biotechnology Perbio (Rockford, IL, USA). The conjugation buffer, (2-[*N*-morpholino] ethanesulfonic acid) Hydrate (MES) was obtained from Sigma-Aldrich.

Human serum albumin (HSA) was obtained from the Business Unit Immune Reagents of our institute (Sanquin). Recombinant human C-reactive protein (rhCRP) was from BiosPacific (Emeryville, CA 94608, USA). Streptavidin-coupled to peroxidase (strept-PO) was purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK). L-C-biotin-*N*-hydroxysuccimide ester was from Pierce. Streptavidin coupled to polymerized horseradish peroxidase was obtained from the Business Unit Immune Reagents (Sanquin).

The mouse monoclonal antibody (mAb) against human CRP (5G4) was produced in our laboratory (Wolbink et al., 1996) and biotinylated (mAb 5G4bt) according to established procedures. mAb M15 against human IgM, coupled to horseradish peroxidase or to biotin, was obtained from the Business Unit Immune Reagents (Sanquin).

2.4. ELISA for anti-Pc IgM antibodies

p-Aminophenylphosphorylcholine was coupled to HSA according to the procedure provided by Pierce (Pierce Biotechnology, www.piercenet.com). Briefly, 4 mg of HSA was coupled to 4 mg of PCh in 0.1 M MES buffer, pH 4.5, containing 2 mg of EDC in a final volume of 1.6 ml. The mixture was incubated for 2 h at RT. The conjugated HSA was then dialyzed against phosphate buffered saline, pH 7.4 (PBS), at 4 °C (Pc–HSA). As a control, glycine instead of PCh was coupled to HSA (glycine–HSA).

Polystyrene microtiter plates (Dynatech, Plochingen, Germany) were incubated o/n at 4 °C with Pc-HSA (2.5 μg/ml) in 0.1 M carbonate/bicarbonate buffer, pH 9.6. Glycine–HSA was also included as a control. Final volume of this as well as of all other steps was 100 μl per well, unless stated otherwise. After washing the plates twice with PBS, residual binding sites were blocked (1 h at RT) with 200 μl per well of PBS containing 2%, w/v, HSA. Human plasma or sera were appropriately diluted in assay buffer (veronal buffer containing 0.1%, w/v, HSA, 2 mM $CaCl_2$, 0.1%, w/v, Tween 20, pH 7.4), and incubated for 1 h at RT. After this and the subsequent incubation steps, the plates were washed with PBS containing 0.1%, w/v, Tween-20. IgM bound to Pc-HSA was quantified with peroxidase-labeled anti-human IgM mAb diluted in assay buffer. Finally, peroxidase activity was visualized by incubation with 3,3′ , 5,5′ -tetra-methyl-benzidine, 100 μg/ml in 0.11 M sodium acetate, pH 5.5, containing 0.003%, v/v, H_20_2. The reaction was stopped after 10 min by addition of 2 M H_2S0_4, and the absorbance at 450 nm was measured in a Titertek plate reader.

Dilutions of a pool of normal plasma, obtained from 40 healthy volunteers, were used to generate a standard curve in each microtiter plate. This standard was arbitrarily proposed to contain 100 U per ml of anti-Pc IgM antibodies. Results with plasma samples

were related to this standard and expressed as U/ml of anti-Pc IgM.

The specificity of the binding of anti-Pc IgM to Pc-HSA was determined by competition immunoassay. The standard curve was pre-incubated with increasing amounts of the competitors (Pc, PE, and PS) or glycine–HSA. After 1-h incubation, the standard with or without competitors was added to the Pc-HSA-coated plates and tested as described above. As another control, four normal sera were diluted 1 to 2 in VB, and absorbed batchwise onto PCh-Sepharose gel (1 volume of 1:2 diluted serum, with 1 volume of Sepharose suspension (capacity 5–11 mg of human CRP per ml gel, 5 ml gel and 5 ml buffer) o/n at 4 °C. Glycine-Sepharose was used as a negative control. The supernatants were then tested in the ELISA.

2.5. Binding of CRP to Pc-HSA-coated plates

Plates with Pc-HSA were prepared as described above, and incubated with recalcified NHP or rhCRP diluted in assay buffer (see previous paragraph) for 60 min at RT. After a washing procedure, bound CRP was detected by incubation for 60 min with biotinylated mAb 5G4 against human CRP (mAb 5G4bt), diluted in assay buffer followed by a subsequent incubation with polymerized peroxidase dissolved in veronal buffer containing 2 mM $CaCl_2$ and 2%, v/v, milk (Campina, The Netherlands). Finally, peroxidase activity was visualized with tetra-methyl-benzidine as described above. As a control, sample incubations were prepared in the presence of 10 mM EDTA.

2.6. Quantification of total IgM

The concentration of total IgM was determined by nephelometry (Behring Nephelometer Analyzer, Marburg, Germany), according to standard procedures.

2.7. ELISA for human CRP

CRP concentration was determined by ELISA as described (Wolbink et al., 1996). Briefly, polyclonal rabbit anti-human CRP (KH61) was used as capture abs and mAb 5G4bt was used as the detecting antibody. Results were obtained by reference to a standard from Behringwerke (Marburg, Germany).

2.8. Binding of IgM to apoptotic cells

Levels of IgM binding to apoptotic cells were assessed with FACS using Jurkat cells as previously described (Zwart et al., 2004). Briefly, Jurkat cells were made apoptotic by incubation with etoposide (Sigma). Cells were washed with serum-free medium and incubated with 10%, v/v, recalcified human plasma for 30 min. IgM binding to the cells was assessed by incubation with biotin-labeled anti-human IgM monoclonal antibody. Results were analyzed by flow cytometry, and expressed as mean fluorescence intensity (MFI). Plasma samples were also tested with vital Jurkat cells, as a control. Under the conditions used, binding of IgM to these vital cells was negligible.

2.9. Statistical analysis

Data were analyzed with the GraphPad software Prism (San Diego, CA, USA). The distribution of anti-Pc IgM in healthy donors was analyzed using a normality test. Groups of data were compared with repeated measures ANOVA. The variation of levels of anti-Pc IgM and CRP was evaluated by comparing intra-individual variation over time with the paired Student's *t*-test (two-tailed). Correlations between parameters were assessed by estimating Spearman's rank correlation coefficient. A two-sided p-value <0.05 was considered to indicate statistical significance.

3. Results

3.1. Quantification of anti-Pc IgM with ELISA

To quantify natural IgM antibodies against phosphorylcholine in humans, an ELISA was developed using PCh coupled to HSA as capture antigen on the plates. The coating antigen was validated by assessing binding of CRP, which has a similar specificity as anti-Pc IgM (Tillet and Francis, 1930; Thompson et al., 1999; Volanakis and Kaplan, 1971; Chang et al., 2002). Upon incubation of the plates with rhCRP or NHP, calcium-dependent binding of CRP was observed to Pc-HSA plates but not to glycine–HSA plates (data not shown). Thus, Pc-HSA was probably a suitable antigen for anti-Pc IgM. This was further assessed by incubation of Pc-HSA plates with serial

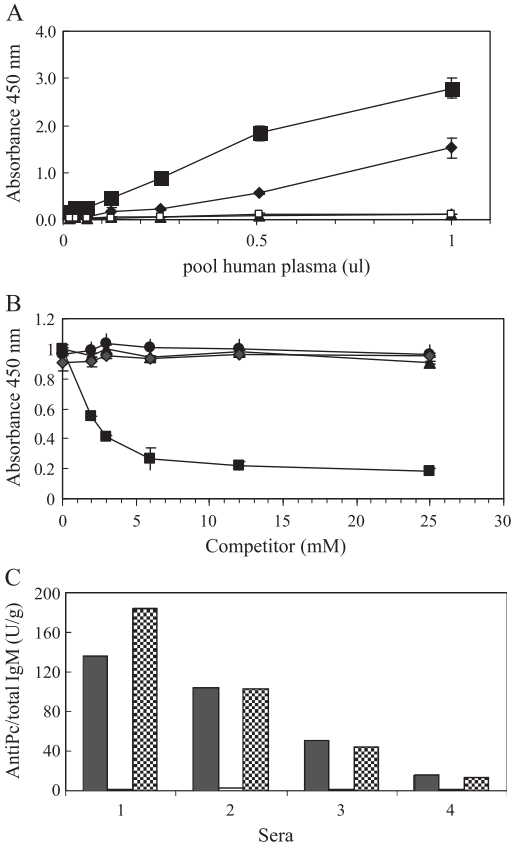

Fig. 1. Specificity of the ELISA for anti Pc IgM antibodies. (A) Microtiter plates were coated with Pc-HSA (■) glycine–HSA (♦) HSA (▲) or coating buffer (□). Dilutions of pooled normal human plasma were then added to the plates. Bound IgM was detected using peroxidase labeled anti-human IgM mAb. Peroxidase activity was visualized with TMB. (B) Pooled normal human plasma was pre-incubated for 1 h with Pc (■), PE (▲), PS (●) or glycine–HSA (♦), and tested in the ELISA. (C) Four sera were absorbed onto Pc-Sepharose and tested in the ELISA. Bars represent the amount of specific IgM against Pc relative to the total IgM (U/g) in the sera absorbed with Pc-Sepharose (open bars), glycine-Sepharose (black chequered bar), or without absorption (black bar). Results in the figures are the means of triplicate determinations. The experiments were repeated twice with similar results.

dilutions of NHP followed by detection of bound IgM with peroxidase–labeled anti human IgM mAb. A dose-dependent binding of IgM was observed (Fig. 1A). In general, IgM is notorious for antigen non-specific sticking to solid phases. Hence, we included a number of controls to rule out the possibility that the observed binding of IgM to Pc-HSA was specific. Binding of IgM to HSA- or non-coated plates upon incubation with dilutions of NHP was negligible. However, significant binding of IgM to glycine–HSA was observed, though this binding was less than that to Pc-HSA-coated plates (Fig. 1A). We did competition experiments to further substantiate the specificity of the ELISA for anti-Pc IgM. Binding of IgM to Pc-HSA-coated plates was almost completely inhibited in the presence of increasing concentrations of Pc during the sample incubation, whereas PE, PS or glycine–HSA had no effect (Fig. 1B). Conversely, binding of IgM to glycine–HSA was inhibited by glycine–HSA but not by Pc, PE or PS (data not shown). In addition, four sera absorbed onto PCh-Sepharose yielded negative results in the ELISA with Pc-HSA-coated plates, whereas the same sera absorbed onto glycine-Sepharose exhibited unaffected IgM binding to Pc-HSA-coated plates (Fig. 1C). Thus, these experiments together demonstrated the specificity of the ELISA with Pc-HSA-coated plates for anti-Pc IgM.

We next tested the effect of the method of blood collection, and of freezing and thawing of samples, on levels of anti-Pc IgM as measured with the ELISA. In Table 1 it can be seen that anti-Pc IgM levels were similar in plasma samples collected in EDTA, sodium citrate or in sodium heparin, as well as in serum samples. Moreover, Table 1 also shows that five additional cycles of freezing at −70 °C and thawing did not affect levels of anti-IgM. We also tested with plasmas from 10 different donors whether binding of IgM in the Pc-HSA ELISA was calcium-dependent. The results showed no difference in levels when

Table 1
Repeated freezing/thawing has no effect on levels of anti-Pc IgM in plasma or serum samples

Sample	t1	t6
EDTA	141.91±28.63	145.08±28.13
Citrate	128.39±21.74	117.15±19.57
Heparin	139.58±22.17	143.16±24.35
Serum	133.96±23.85	151.13±29.43

Values represent mean±S.E.M. of the anti-Pc IgM concentration in U/ml of six healthy donors.
EDTA, citrated or heparin plasma or serum samples were frozen at −70 °C and thawed one (t1) to six (t6) times, and then tested in ELISA.

EDTA was present during the sample incubation step (data not shown).

3.2. Variation of anti-Pc IgM levels in healthy donors

To quantify results obtained with plasma samples, a pool of plasma samples from healthy donors was arbitrarily assigned 100 U of anti-Pc IgM per ml. Results with samples to be tested were then compared with this plasma pool and expressed as U/ml. In 40 healthy donors, the median concentration of anti-Pc IgM was 108 U/ml, the 25th percentile being 55 U/ml, and the 75th percentile 162 U/ml. The lowest concentration of anti-Pc IgM in these healthy donors was 3 U/ml and the highest was 469 U/ml. Thus, levels of anti-Pc IgM in the healthy donors differed considerably, i.e. more than 100-fold. The distribution of anti-Pc IgM in the donors according to age is shown in Fig. 2A, and that according to sex is shown in Fig 2B. Younger or older persons had similar levels of anti-Pc IgM. Moreover, men had similar levels as women. To estimate variation of anti-Pc IgM levels over time, plasma samples from 20 healthy donors were collected weekly during a period of 6 weeks, and tested for anti-Pc IgM with the ELISA. Small fluctuations in anti-Pc IgM levels were observed (coefficient of intra-individual variation over time: 6–25%; Fig. 3A). For comparison, CRP plasma levels were also measured in the same samples and appeared to differ significantly ($p<0.0001$) more during the observation period of 6 weeks than anti-Pc IgM levels (coefficient of intra-individual variation over time for CRP: up to 114%; Fig. 3B). These differences in variation of plasma levels of anti-Pc IgM CRP were not explained by different reproducibility of the assays, since the coefficient of inter-assay variation was less than 10% for either assay.

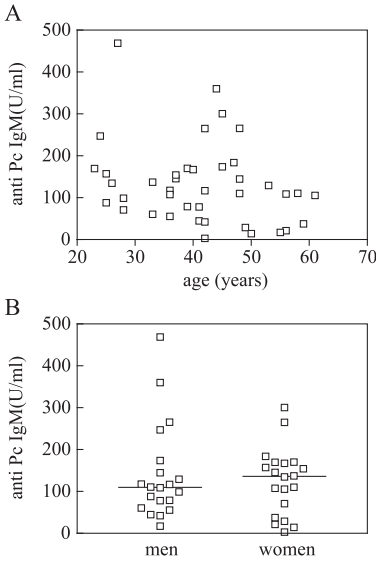

Fig. 2. Distribution of anti-Pc IgM in healthy controls according to age (A) or sex (B).

Because of the functional similarities (specificity for PC; complement activation) between anti-Pc IgM and CRP, we studied the correlation between levels of either parameter in the healthy individuals. However, the levels of anti-Pc IgM and CRP, did not correlate (Spearman's r: 0.115, p=0.2429). Furthermore, we also assessed the relationship between anti-Pc IgM and total IgM concentration. These parameters showed only a borderline correlation (Spearman's r 0.44; p=0.051).

3.3. Anti-Pc IgM in healthy donors correlate with IgM binding to apoptotic cells

IgM with specificity for Pc has been shown to bind to apoptotic cells (Kim et al., 2002; Chang et al., 1999, 2002; Shaw et al., 2000). In order to examine the relation between levels of natural IgM abs as measured with Pc-HSA-coated plates, and IgM binding to apoptotic cells, 30 sera were tested both in the anti-Pc IgM ELISA as well as for binding of IgM to apoptotic Jurkat cells. As seen in Fig. 4, anti-Pc IgM correlated positively with the amount of IgM that bound to the apoptotic cells ($p<0.0001$).

3.4. Circulating levels of anti-Pc IgM antibodies decrease during tissue damage

We postulated that during tissue damage, neo-antigens are exposed on the membranes of jeopardized cells, which may be recognized by the anti-Pc IgM antibodies. Consequently, a decrease in plasma levels of anti-Pc IgM is expected in situations of sufficient tissue damage. To test this hypothesis, the kinetics of anti–Pc IgM in plasma samples from five patients undergoing isolated limb perfusion with TNFα because of sarcoma or melanoma was determined. In

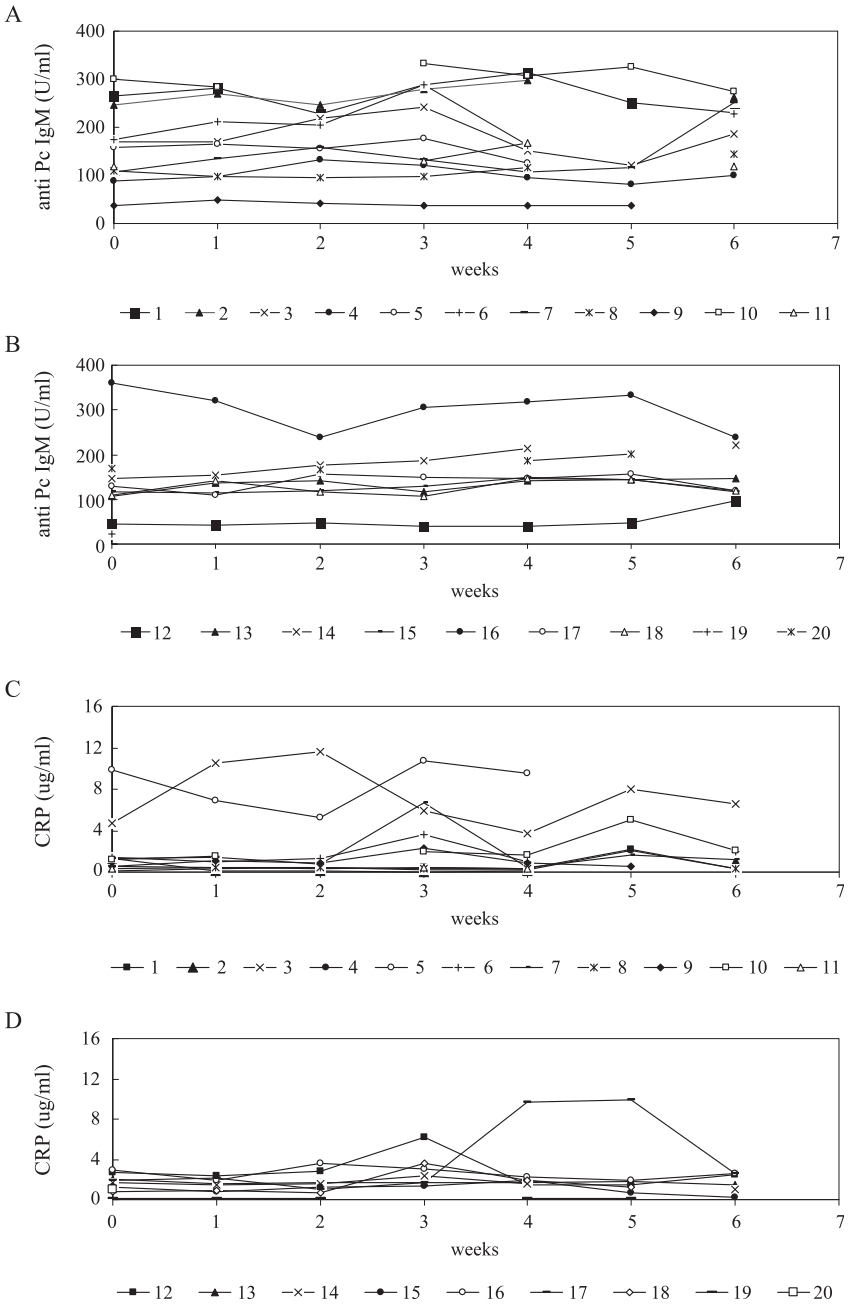

Fig. 3. Fluctuation of individual levels of anti-Pc IgM over time. Anti-Pc IgM (A and B) and CRP concentrations (C and D) were measured in plasma obtained weekly from healthy controls.

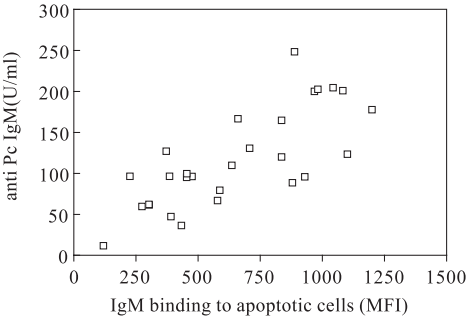

Fig. 4. Relationship between anti-Pc IgM and IgM binding to apoptotic cells in healthy individuals. Anti-Pc IgM and IgM binding to apoptotic Jurkat cells were measured in samples from 30 healthy donors with ELISA and FACS, respectively. The concentration of anti-Pc IgM is expressed in U/ml and IgM binding to apoptotic cells is presented as mean fluorescence intensity (MFI).

all patients, regression of the tumor was observed, and was accompanied by high levels of several pro-inflammatory cytokines such as IL-6 (data not shown). The anti-Pc IgM concentration was related to total IgM levels in order to adjust for hemodilution. A moderate decrease of anti-Pc IgM levels was observed in all patients at 24 h after perfusion ($p>0.05$). At 48 h after perfusion, this decrease was up to 60% and had become significant ($p<0.05$; Fig. 5).

4. Discussion

Some decades ago, anti-Pc IgM was considered to constitute a first-line defense against microorganisms. More recently, interest in these antibodies was renewed because of their potential involvement in processes such as atherosclerosis (Binder et al., 2003; Nilsson et al., 1997; Palinski et al., 1995, 1996; Wu et al., 1998) or removal of apoptotic cells and cellular debris (Kim et al., 2002). As a consequence, levels of anti-Pc IgM may constitute a risk marker for human diseases such as cardiovascular disease (Rose and Afanasyeva, 2003). Here we describe a specific and reproducible ELISA for the quantification of anti-Pc IgM. Levels of these antibodies differed by more than 100-fold among healthy donors, whereas the intra-individual variation over time was limited. Furthermore, anti-Pc IgM levels correlated well with IgM binding to apoptotic Jurkat T cells and decreased in patients with sarcoma or melanoma upon isolated limb perfusion with TNF-α.

Quantification of IgM with ELISA in general is associated with an increased risk of non-specific binding of IgM to the plates. However, the ELISA for the quantification of natural anti-Pc IgM in humans described here was considered specific since the signal was abolished when plasma was pre-incubated with Pc, but not PE or PS. In addition, pre-absorption of sera with Pc-Sepharose reduced IgM binding in the ELISA. Furthermore, pre-incubation of plasma with modified albumin (glycine–HSA) did not affect the response of plasma samples in the anti-Pc IgM ELISA, ruling out the possibility that IgM detected in this assay had interacted with the chemical linker used to prepare Pc-albumin. Thus, the IgM detected in the ELISA was IgM specifically binding to Pc. A limitation of the use of Pc-albumin as a coating antigen in the ELISA is that Pc conjugated

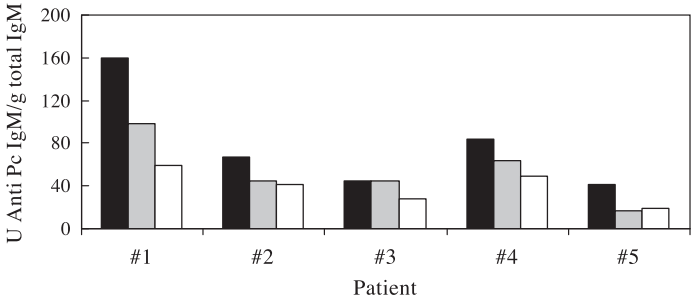

Fig. 5. Levels of anti-Pc IgM before and after limb perfusion with TNFα. Isolated limb perfusion with TNFα was performed in five patients with sarcomas during 3.5 h. Systemic blood samples were taken before (t0, black bars) and after perfusion, 24 h (grey bars) and 48 h (open bars). The concentration of anti-Pc IgM was adjusted to total IgM concentration quantified by nephelometry as described in the Materials and methods.

to HSA may not resemble Pc presented by injured cells. However, CRP, which has a well-known specificity for Pc (Volanakis and Kaplan, 1971; Thompson et al., 1999) and is able to bind to apoptotic and injured cells (Gershov et al., 2000; Chang et al., 2002), easily bound to the Pc-HSA. Thus, Pc conjugated to HSA resembled Pc exposed on apoptotic or injured cells. This was confirmed by the observation that levels of anti-Pc IgM in healthy donors correlated with IgM binding to apoptotic cells.

Studies on the relationship between biochemical markers and cardiovascular events are more credible when markers to be measured in blood samples are stable during repeated freezing and thawing. As with anticardiolipin IgM (Brey et al., 1994), we found no significant difference in mean concentration of anti-Pc IgM after repeated freeze–thaw cycles.

Notably, the levels of anti-Pc IgM differed by more than 100-fold among healthy donors, though there was no relationships between these levels and age or sex. In animal models for atherosclerosis anti-Pc IgM increase over time (Binder et al., 2003). Our data do not support the notion that anti-Pc IgM in humans is increased in atherosclerosis since in that case, some relationship between plasma levels and age would be expected. Conversely, anti-Pc IgG1 was significantly higher in children than in adults (Freijd et al., 1988). In addition, anti-Pc IgM levels did not correlate with total IgM concentration, as has been found earlier for anti-Pc IgG (Freijd et al., 1988). We speculate that the inter-individual differences in anti-Pc IgM levels among healthy donors reflect genetic variations as well as exposure to different microbes during life.

Anti-Pc IgM and CRP share similarities in biological function in that they can bind to Pc exposed on injured cells and activate complement (Gershov et al., 2000; Pepys and Hirschfield, 2003). Nevertheless, plasma levels of these moieties did not correlate with each other. Hence, one can speculate that IgM and CRP constitute two independent mechanisms taking care of injured cells. The relative contribution of anti-Pc IgM and CRP to complement activation could differ between individuals depending of their relative concentration. Indeed, we (Krijnen et al., unpublished observations) have recently shown that the relative amounts of CRP and IgM deposited on cardiomyocytes in infarcted myocardium may differ from patient to patient.

Complement activation by apoptotic cells in plasma is mainly mediated by IgM binding to neoepitopes exposed in the membranes of these cells (Kim et al., 2002) (Zwart et al., 2004). Though it has been claimed that most if not all of these IgM antibodies recognize Pc exposed in the lysophosphatidylcholine in the cell membrane (Kim et al., 2002), we have observed that this only holds for some individuals, whereas others also have IgM recognizing additional, unknown epitopes (Ciurana and Hack, in press). In this study, we report a good correlation between the levels of anti-Pc IgM as measured by ELISA and the amount of IgM binding to apoptotic Jurkat cells. This finding is consistent with the notion that part of the IgM binding to apoptotic cells recognizes Pc. The specificity of the rest of the IgM recognizing apoptotic cells is currently under investigation.

To further verify that anti-Pc IgM can bind to apoptotic or damaged cells, we measured plasma levels of these antibodies in patients with sarcoma or melanoma and treated by isolated limb perfusion with TNF-α. Most of these patients respond well to this therapy within a few days (van Ginkel et al., 1996; Koizumi et al., 2003), and exhibit massive death of both malignant cells and endothelial cells of the tumor vasculature. Ischemia and subsequent reperfusion of the perfused limb may also add to cell death. A decrease of anti-Pc IgM relative to total IgM levels was observed in all of the patients tested, in agreement with the notion that anti-Pc IgM can bind to oxidized or hydrolyzed phospholipids of damaged cells. We speculate that this mechanism contributes to the clearance of damaged cells and cellular debris.

In conclusion, we describe a specific and reproducible ELISA for anti-Pc IgM. Levels of this IgM differ by up to 100-fold among healthy persons. Future studies on anti-Pc IgM should delineate its role in human diseases such as cardiovascular disease.

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CHAPTER 6

Relation of IgM antibodies against apoptotic cells and phosphorylcholine to the inflammatory response and infarct size in patients with acute myocardial infarction

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Abbreviations: VB, Veronal buffer saline; sPLA2, secreted Phospholipase A 2; CRP, C reactive protein; SAP, Serum amyloid p component; AMI, Acute myocardial infarction; MFI, Mean fluorescence intensity; LDH, Lactate dehydrogenase; CK, Creatine kinase; PCh, Phosphorylcholine.

In preparation

Abstract

Background: Natural IgM antibodies, particularly anti-phosphorylcholine IgM (anti-PCh IgM), are suspected to be involved in cardiovascular disease as they possibly modulate atherosclerosis or acute myocardial infarction (AMI). Aim: To assess the relationship between plasma levels of anti-PCh IgM and IgM that binds to damaged cells, with infarct size or post-infarct inflammatory responses in patients with AMI. Methods: Plasma samples from 50 patients with AMI and 46 healthy controls were analysed. IgM binding to damaged cells was measured by incubating plasma samples with apoptotic Jurkat cells with subsequent detection of bound IgM by fluorescence activated cell sorter (FACS). Anti-PCh IgM was measured with a specific ELISA. The post-infarct inflammatory response was quantified by measuring C-reactive protein (CRP), secretory phospholipase A2 (sPLA2), IL6, IL8 and activated complement. Results: On admission, patients with AMI had similar levels of IgM binding to apoptotic cells but lower levels of anti-PCh IgM, also when corrected for hemodilution, than healthy controls. These levels were constant during 48 hours. To analyse the relation of IgM species to apoptotic cells and inflammatory or clinical parameters, patients with levels above the median were compared to those with levels below the median. Patients with higher levels of IgM binding to apoptotic cells had similar inflammatory responses and infarct size as those with lower levels. Patients with higher levels of anti-PCh IgM, however, had larger infarcts as assessed with ECG, and a more pronounced response of the acute phase protein sPLA2. Conclusion: Plasma levels of natural IgM, in particular anti-PCh IgM, may modify inflammatory responses and infarct size in patients with acute myocardial infarction.

Introduction

Patients with acute myocardial infarction (AMI) frequently develop fever and have increased plasma levels of cytokines and acute phase proteins such as C-reactive protein (CRP) and secretory phospholipase A2 (sPLA2) in the first days following the onset of infarction. Thus, myocardial infarction induces an inflammatory response in the ischemic tissue which may lead to systemic symptoms. Indeed expression of cytokines and infiltration of polymorphonuclear granulocytes (PMN) occurs in the jeopardized myocardium during infarction [1-4]. Furthermore, acute phase proteins such as CRP and sPLA2 are deposited in the infarcted myocardium and the complement system is activated locally [5-7]. Cardiomyocytes in the ischemic area may show evidence for apoptosis [8]. To what extent this post-infarct inflammatory response in the heart contributes to the infarct size in humans is unknown. Animal studies suggest that this contribution may be substantial [9].

Though local ischemia obviously is the primary stimulus for the inflammatory changes ensuing in the infarcted myocardium, the molecular mechanisms explaining the link between ischemia and inflammation are far from clear. Possibly, changes in membrane phospholipids such as oxydation, hydrolysis and membrane flip-flop due to the formation of oxygen radicals, the decrease of intracellular ATP and the increase of intracellular calcium, stimulate the binding of extracellular proteins such as sPLA2 and CRP [10;11], which in turns activate complement thereby triggering inflammation. Some evidence suggests that also natural IgM antibodies may be among proteins that bind to altered phospholipids in the membranes of ischemic cells, and via subsequent activation of complement contribute to the post-infarction inflammatory response. For example, studies in knock-out mice have convincingly shown that natural IgM is involved in ischemia-reperfusion injury by binding to ischemic endothelial cells and by triggering reperfusion-induced complement activation [12]. The specificity of this IgM is still unknown. In immunohistochemical studies we recently observed that IgM becomes deposited in the ischemic myocardium during infarction in humans who died from AMI. This IgM is co-localized with CRP and activated complement [13]. Thus, in humans IgM, in addition to CRP [5;6] and possibly other molecules, may contribute to local complement activation in the ischemic myocardium. The nature of this IgM is also unknown.

A number of studies have shown that IgM present in normal serum can bind to apoptotic cells [14;15], including natural IgM against phosphorylcholine (anti-PCh IgM) [16]. In the present study we tested the hypothesis that IgM that binds to damaged cells and or anti-PCh IgM may enhance the post-infarct inflammatory response in patients with AMI. To test this hypothesis, we developed an assay to measure plasma IgM that binds to apoptotic cells, and we used this assay, as well as an ELISA for anti-PCh IgM, to measure plasma levels of this IgM in patients with AMI. These levels were compared to those in healthy controls and were also related to parameters for cardiac damage and of inflammatory mediators such as IL6, IL8 and activated complement.

Patients, material and methods

Blood samples

Blood samples from 50 patients with AMI were collected in 10 mM EDTA, final concentration, at various time points after admission to the hospital. Samples were centrifuged at 1300 g after which the supernatant was stored in aliquots at -70ºC until tested. All patients fulfilled the criteria for AMI, which included typical chest pain, typical electrocardiographic

abnormalities in combination with elevated cardiac markers such as creatine-kinase (CK) [17]. Infarct size was calculated from the cumulative release of lactate dehydrogenase (LDH) or CK [18;19], and also from electrocardiographical infarct scores (Selvester scores) in patients with a first AMI before therapy [20;21]. Patients had participated in earlier studies on the role of complement in AMI. All patients had given informed consent. The study was approved by the Medical Ethical committee of the VU Medical Centre.

Plasma samples from a group of 46 healthy donors were collected and processed in the same way. In addition, serial samples were obtained from 5 healthy donors weekly for a period of 6 weeks. During this period none of the volunteers suffered from an intercurrent illness.

Proteins and antibodies

Anti-human IgM monoclonal antibody (mAb) MH-15 (IgG1 subclass) was obtained from Sanquin, Business Unit Reagents (Amsterdam, The Netherlands). The mAb was biotinylated with LC-biotin-n-hydroxysuccinimide ester (Pierce, Rockford, IL) according to the manufacturer's instructions. AnnexinV-FITC and propidium iodide (PI) were obtained from Bender Med System (Vienna, Austria) and streptavidin-allophycocyanin (Strep-APC) conjugate was obtained from BD Biosciences Pharmingen (San Diego, CA). Human serum albumin (HSA), purchased at Sanquin Business Reagent and p-aminophenylphosphoryl-choline (Sigma Chemicals Co., St Louis, MO) were coupled following a protocol described earlier [22].

Assay for IgM binding to late apoptotic cells (apo-IgM)

Jurkat cells were cultured in IMDM supplemented with 5%, v/v, heat-inactivated foetal calf serum (Bodinco, Alkmaar, The Netherlands), 20 µg/ml human apo-transferrin (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere (5%, v/v, CO_2 / 95% air). Apoptosis was induced in Jurkat cells by incubation in 96 wells round-bottom plates at 2.5 $x10^5$ cells per well in serum-free IMDM containing 100 µM etoposide (Sigma) for 48 hours at 37ºC. After apoptosis induction, cells were washed with serum-free culture medium to remove etoposide and incubated with various amounts of plasma in veronal buffered saline, pH 7.4 (VB), containing 10 mM $CaCl_2$ and 2 mM $MgCl_2$ (VB++), final volume 100 µl. Plasma samples from patients were tested in this system at a concentration of 5%, v/v, unless otherwise indicated. After an incubation for 30 minutes at 37°C, cells were washed thrice with Hepes buffer containing calcium (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, $CaCl_2$ 1.8 mM and $MgCl_2$ 1 mM, pH 7.4) and incubated with biotin-labelled monoclonal anti-IgM (5 µg per ml in 50 µl of hepes buffer, final volume) for 30 minutes at 4°C in darkness. After a second washing procedure, cells were incubated with streptavidin-APC (1 to 750 diluted) or annexin-V-FITC (1 to 100 dilution) in 100 µl final volume for 30 minutes at 4°C in darkness. After washing with Hepes buffer, cells were resuspended in Hepes buffer containing calcium as well as PI (at a final concentration of 500 ng per ml). Results were analysed by flow cytometry, and expressed as mean fluorescence intensity (MFI). Data were stored in Cellquest acquisition program and analysed with WinMDI 2.8 program.

ELISA for anti-phosphorylcholine IgM

IgM antibodies directed against phosphorylcholine (PCh) were measured by specific ELISA as described [22]. Briefly, p-aminophenylphosphorylcholine coupled to human serum albumin (PCh-HSA; 2.5 µg/ml in 0.1 M carbonate/bicarbonate buffer, pH 9.6) was coated onto

polystyrene microtiter plates (Dynatech, Plochingen, Germany) overnight at 4°C. After washing the plates twice with PBS, residual binding sites were blocked (1 hour at room temperature) with 200 µl per well of phosphate buffered saline, pH 7.4 (PBS) containing 2 %, w/v, HSA. Human plasma or sera were appropriately diluted in assay buffer (veronal buffer containing 0.1 %, w/v, HSA, 2 mM $CaCl_2$, 0.1 %, w/v, Tween 20, pH 7.4), and incubated for 1 hour at room temperature. Thereafter, the plates were washed with PBS containing 0.1 %, w/v, Tween-20. IgM bound to PCh-HSA was quantified with peroxidase-labelled anti-human IgM mAb diluted in assay buffer. Finally, peroxidase activity was visualized with tetra-methyl-benzidine. Dilutions of a pool of normal plasma, obtained from 40 healthy volunteers, were used as standard curve in the assay. This standard was arbitrarily said to contain 100 U per mL of anti-PCh IgM antibodies. Results of plasma samples were related to this standard and expressed as U of anti-PCh IgM per mL.

Biochemical and inflammatory parameters
IgM concentration was assessed with a nephelometric assay. Secretory phospholipase A_2 (sPLA2), CRP, interleukins-6 and -8 (IL6 and IL8, respectively), and activated complement fragments C4b/c and C3b/c were determined with specific ELISA as described earlier [23-27]. Note that the ELISAs do not discriminate between C4b, C4bi or C4c, and C3b, C3bi and C3c, and that the activation products detected by these assays are hence referred to as C4b/c and C3b/c, respectively. Results were expressed as µg/L (sPLA2)[23], mg/L (CRP) [24], ng/L (IL8 and IL6) [25;26] and nmol/L (C3b/c and C4b/c) [27]. LDH and CK concentrations were determined in the routine clinical chemistry laboratorium of the VU Medical Centre.

Analysis of data
Data were analysed with Graph Pad Instat® (version 3.0). Distribution of data was analysed with the method of Kolmogorov and Smirnov. When normally distributed, data were analysed with Student's t test or one-way analysis of variance (ANOVA) multiple comparison test with Bonferroni. Correlation between parameters was assessed by calculating the Pearson's correlation coefficient. In case of non-normal distribution, Mann-Whitney's and Kruskal-Wallis with Dunn's multiple comparison tests were used to assess differences between groups. A P-value <0.05 was considered to represent a significant difference or correlation.

Results

Patients included
The patients with AMI, 8 females and 42 males, included in the analysis had a median age of 60 years, range 34 to 76 years. Seventy % of the patients were treated with thrombolytic agents, 20% with acute percutaneous transluminal coronary angioplasty. Twenty-four patients had a first infarction; the others had suffered from a myocardial infarction before. None of the patients had an underlying illness, none died during the observation period.

IgM binding to apoptotic cells
IgM that binds to damaged cells was measured by incubating apoptotic Jurkat cells with plasma samples, and detection of bound IgM with FACS [15]. A triple staining procedure allowed the determination of specific IgM that bound to the late apoptotic population, which was identified by its characteristic side and forward scatter dot plot (figure 1A), and by its capacity to bind annexinV and propidium iodide (figure 1B). IgM bound to the cells was then

assessed with the third marker, streptavidin-APC that bound to biotinylated anti-IgM (figure 1C). Thus, the mean fluorescence intensity (MFI) of the streptavidin-APC of the gated population was determined as a measure for IgM binding to apoptotic cell (anti-apo IgM). Cells not incubated with plasma were used as control for aspecific staining (figure 1D). IgM binding to apoptotic cells appeared to vary among donors. For example, figure 1D shows the results, expressed as MFI, obtained when plasma samples from 2 different healthy donors were tested.

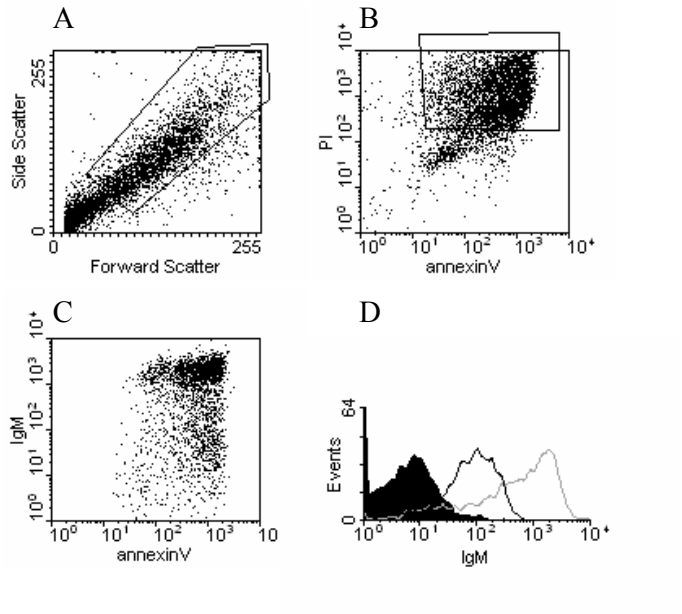

Figure 1: IgM binding to apoptotic cells. Apoptosis was induced in Jurkat cells as described in material and methods. *Cells were subsequently incubated with plasma. After selection of the late apoptotic population (encircled) by specific side and forward scatter (A), and identification of apoptotic cells with annexin V and propidium iodide positive, (encircled) (B), IgM bound to the cells was detected with biotinylated anti-IgM mAb and streptavidin-APC (C). (D) Histograms showing IgM binding to apoptotic cells using 2 plasma samples from fluorescence intensity (MFI). Cells incubated without plasma are indicated by the filled histogram.*

To get insight into the variation in time of levels of anti-apo IgM, plasma samples were obtained weekly from 5 healthy lab donors during a period of 6 weeks, and measured for anti-apo IgM. Intra-individual levels of anti-apo IgM hardly varied during this period with a variation coefficient (CV) of 11 % ± 1.0, (figure 2). Previously we have observed that the variation of levels of anti-PCh IgM in time is also limited in healthy individuals [22]. In order to reduce assay variation as much as possible, all plasma samples were tested within the same experiment.

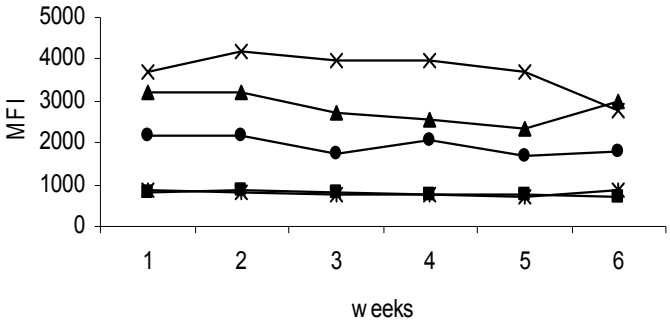

Figure 2: Variation of IgM binding to apoptotic cells in time. *Apoptotic Jurkat cells were incubated with plasma samples taken weekly from 5 healthy donors for a period of 6 weeks. IgM binding to apoptotic population was detected as described in material and methods and expressed as mean fluorescence intensity.*

Anti-apo IgM and anti-PCh IgM in the AMI patients

Levels of anti-apo IgM were determined as described above in blood samples from patients with AMI or from healthy controls (figure 3). Levels were somewhat lower in the AMI patients as compared to those in the healthy controls (230 MFI ±34, mean ±sem, n=50, versus 264 MFI ±24, n=46; $p<0.05$). Moreover, levels of anti-apo IgM 48 hours after the onset of complaints had further decreased compared to those on admission (169 MFI ±15 versus 230 MFI ±34, n=45; $p<0.001$). These differences in levels between patients and controls, however, were lost when levels were corrected for hemodilution by calculating the ratio of anti-apo IgM to total IgM (patients t=0: 93 ±8 and 114 ±10 at t=48, versus healthy controls 86 ±5, figure 4A).

In addition to anti-apo IgM we also determined the concentration of anti-PCh IgM by ELISA as described in materials and methods. Similarly as for anti-apo IgM, values were corrected for hemodilution by estimating the ratio of anti-PCh IgM to total IgM. Although there was no significant difference between the ratios of the patients on admission versus those at 48 hours (84 ±7, mean ±sem, versus 87 ±10, respectively), the ratio at either time point was significantly lower than that in the control group of healthy donors (141 ±17, respectively $p<0.01$ and $p<0.001$, figure 4B).

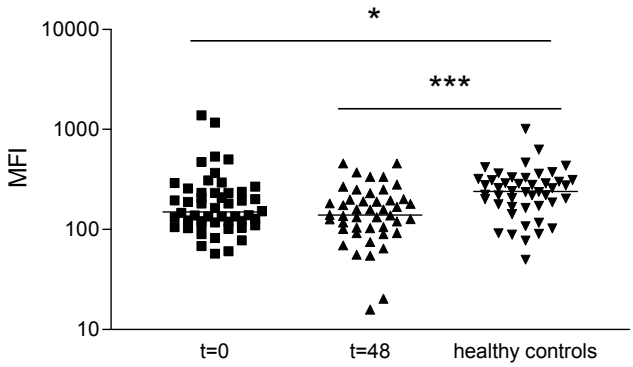

Figure 3.

Figure 3: Anti-apo IgM in patients with myocardial infarction and controls. *Apoptotic Jurkat cells were used to measure levels of anti-apo IgM. Plasma samples from patients on admission (t=0; n=50) or 48 hours later (t=48; n=45), and from healthy controls (n=46) were tested for anti-apo IgM as described in material and methods. Levels are expressed as MFI, data were analysed with Kruskal-Wallis and Dunn's multiple comparison test. *P<0.05 and ***P<0.001.*

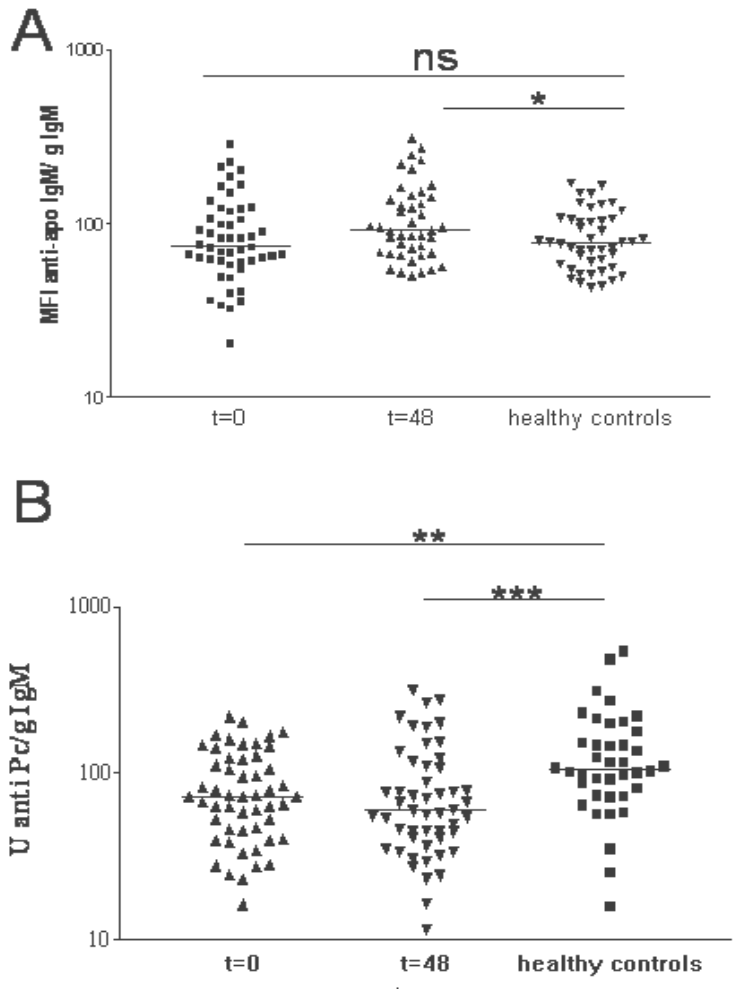

Figure 4: Anti-apo IgM (A) and anti-PCH IgM (B) in patients with myocardial infarction. *Levels were corrected for hemodi-lution by assessing the ratio of anti-apo IgM to total IgM (A) and of anti-PCh IgM to total IgM (B). Levels on admission (t=0) and at 48 hours (t=48) are given for the patients, as are the ratios measured in healthy controls. Data were analysed with Kruskal-Wallis and Dunn's multiple comparison test. *P<0.05, **P<0.01 and ***P<0.001.*

Relation of anti-apo IgM or anti-PCh IgM with infarct size

Infarct size in the patients was estimated from the course of LDH and CK-MB. This course was quantified by calculating a cumulative concentration over the first 48 hours after the onset of complaints [18]. To establish the relation of levels of anti-apo IgM or anti-PCh IgM to infarct size, we compared the cumulative release of LDH or CK-MB in patients with anti-apo IgM levels lower or higher than the median value at the onset of the infarction (Table 1 and figure 5 A, B).

No significant difference in cumulative release of LDH or CK was observed between the groups. The cumulative release of these cardiac enzymes during the first 48 hours in patients having high anti-PCh IgM on admission was higher than that in the patients with low levels of anti-PCh IgM (Table 1 and figure 5 D, E), although the difference between the groups did not reach a statistical significance. In patients without a previous infarction infarct, size can also be measured from the electrocardiographic criteria, as indicated by the Selvester score. Twenty-four patients could be evaluated in this way. There was no difference in Selvester score of patients on admission with high or low levels of anti-apo IgM on admission (figure 5C). However, the score was significantly higher in patients with high levels of circulating anti-PCh IgM on admission, as compared to those with low levels (figure 5F).

IgM level / Marker	Low anti-apo IgM	High anti-apo IgM		Low anti-PCh IgM	High anti-PCh IgM	
LDH (U/L)	552.9 ±85.9 (n=22)	524.5 ±80.1 (n=23)	*P=0.81*	462.8 ±66.7 (n=23)	617.4 ±94.6 (n=22)	*P=0.19*
CK (U/L)	595.4 ±114.4 (n=22)	566.3 ±82.7 (n=21)	*P=0.84*	521.4 ±77.5 (n=21)	638.2 ±116.4 (n=22)	*P=0.41*
ECG (selvester score)	28.6 ±3.2 (n=13)	27.6 ±2.9 (n=11)	*P=0.81*	23.2 ±3.1 (n=11)	32.3 ±2.5 (n=13)	**P=0.03*

Table 1: Relation of anti-apo IgM or anti-PCh IgM on admission to cardiac markers. *Anti-apo IgM levels were determined with apoptotic Jurkat cells and anti-PCh IgM on admission was determined by ELISA. Patients with low (<median) or high (>median) levels of anti-apo IgM or anti-PCh IgM were compared regarding cumulative concentration over 48 hours of creatine kinase (CK), lactate dehydrogenase (LDH) and their electrocardiographic (ECG) score. Data are given as mean value ± sem, and the difference between groups was analyzed with two-tailed Student's t test when they appeared to be normally distributed.*

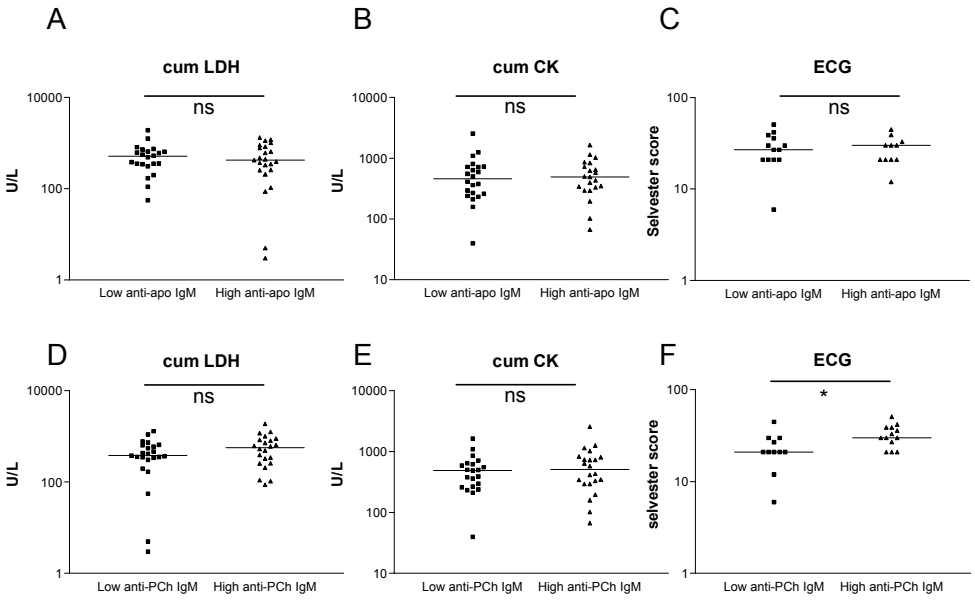

Figure 5: Relation between apo-IgM, anti-PCh IgM and infarction size. *(A, B, C): Levels of anti-apo IgM were measured with apoptotic Jurkat cells and expressed as MFI. Patients were said to have low or high levels when they had levels below or above, respectively, the median of the group. Electrocardiographic (ECG) results evaluated by cumulative release values of cardiac enzyme over 48 hours LDH (A) and CK (B) expressed as units per litre (U/L) and the Selvester score (C), and A similar analysis was made for anti-PCh IgM (D, E, and F). Data were analysed with Student's t test. ns=non significant, *P<0.05.*

Relation of anti-apo IgM or anti-PCh IgM with post-infarct inflammatory response

A number of parameters including the acute phase proteins CRP and sPLA2, the cytokines IL6 and IL8, and activated complement fragments C4b/c and C3b/c, were determined to assess the inflammatory response in the patients. The cumulative concentration of the acute phase protein, sPLA2, over 48 hours after admission was comparable between patient groups having low or high levels of anti-apo IgM. However, the patients with increased circulating anti-PCh IgM on admission had a significant increase in their sPLA2 concentration when compared to patients with lower anti-PCh IgM (1031 µg/L ±175 versus 860 µg/L ±308, P=0.03, respectively; Table 2). Pro-inflammatory cytokines IL6 and IL8 concentrations 48 hours after admission were comparable in patients having anti-apo IgM values or anti-PCh IgM below or above the median values (Table 2). The cumulative concentration of CRP over the 48 hours following admission was increased in the patients having both anti-apo IgM and anti-PCh IgM above the median values but did not reach a statistical significance (88 mg/L ± 16 versus 149 mg/L ± 36, p=0.12, in patients with low or high anti-apo IgM, respectively, and 101 mg/L ± 22 versus 146 mg/L ± 34, p=0.26, in patients with low and high anti-PCh IgM, respectively, mean ± sem). Similarly, the activated complement fragments, C4b/c and C3b/c 48 hours after admission were comparable between patients and controls (Table 2).

Marker \ IgM level	Low anti-apo IgM	High anti-apo IgM		Low anti-PCh IgM	High anti-PCh IgM	
$sPLA_2$ (μg/L)	943.8 ±316.0 (n=22)	943.3 ±180.1 (n=23)	*P=0.46*	860.7 ±308.1 (n=23)	1031.0 ±175.1 (n=22)	**P=0.03*
CRP (mg/L)	87.6 ±15.5 (n=22)	148.5 ±35.5 (n=22)	*P=0.12*	100.7 ±21.7 (n=23)	145.9 ±33.5 (n=21)	*P=0.26*
C4b/c (nmol/L)	101.2 ±20.6 (n=22)	136.5 ±33.48 (n=23)	*P=0.38*	117.2 ±20.6 (n=22)	125.3 ±35.4 (n=22)	*P=0.85*
C3b/c (nmol/L)	156.7 ±44.5 (n=22)	278.9 ±225.0 (n=22)	*P=0.35*	152.7 ±44.5 (n=22)	288.4 ±224.6 (n=22)	*P=0.39*
IL6 (ng/L)	11.2 ±4.5 (n=22)	10.1 ±2.7 (n=23)	*P=0.33*	11.8 ±4.9 (n=22)	9.7 ±2.0 (n=22)	*P=0.07*
IL8 (ng/L)	12.1 ±2.1 (n=19)	9.1 ±2.1 (n=22)	*P=0.30*	12.9 ±2.3 (n=21)	8.1 ±1.7 (n=22)	*P=0.09*

Table 2: Relation of anti-PCh IgM on admission to inflammatory markers. *Anti-apo IgM levels were determined with apoptotic Jurkat cells and anti-PCh IgM on admission was determined by ELISA. Patients with low (<median) and high levels of anti-apo IgM or anti-PCh IgM (>median) were then compared regarding cumulative concentration over 48 hours of $sPLA_2$, CRP, complement activated products (C4b/c and C3b/c) and cytokines IL6 and IL8. Data are given as mean value ± sem, and were analysed with two-tailed Student's t test as they appeared to be normally distributed. *P<0.05 was considered significant.*

Discussion

Recently, the role of IgM antibodies in cardiovascular disease has received considerable attention. It has been shown to play a role in the course of atherosclerosis [28;29], although their function has not been completely elucidated. Furthermore, our own studies show that IgM antibodies localize in infarcted human myocardium together with activated complement and CRP [13]. Co-localization with CRP, which can bind to PCh, suggests this IgM in infarcted myocardium in part is directed against PCh. Interestingly, IgM against PCh can bind to apoptotic cells [16]. In the present study, we measured IgM binding to apoptotic cells, as well as anti-PCh IgM in patients with AMI and found that levels of the latter antibodies on admission were related to the post-infarct response as well as to infarct size.

Though anti-PCh IgM has been found to bind to apoptotic cells [16;30], competition experiments with fluid-phase PCh indicate that not all IgM binding to these cells is directed against PCh [31]. Hence, to cover other specificities of IgM binding to apoptotic cells, we developed an assay in which apoptotic Jurkat cells are used as solid phase antigen and IgM bound to the cells is detected with FACS. The variability of levels of the IgM binding in this assay, in time was limited in healthy individuals (see figure 2) indicating a limited variation of

levels in individuals over time. Notably, in an earlier study we have also observed a similar limited variation of levels of anti-PCh IgM over time [22].

Levels of IgM binding to apoptotic cells at first glance were lower in the patients than in the healthy controls. However, as patients with AMI receive (intravenous) fluid infusions, we decided to correct levels for hemodilution by calculating the ratio to IgM. Indeed ratios of anti-apo IgM and total IgM appeared to be similar in patients versus healthy controls. Hence, these data did not support the possiblity that binding of this IgM to the ischemic myocardium had resulted in lower levels in the circulation. In contrast, levels of anti-PCh IgM, even when corrected for total IgM, were significantly lower in the patients on admission as compared to levels in healthy controls. Presumably this different behaviour of anti-PCh IgM versus anti-apo IgM reflects that absolute levels of the former are lower than those of the latter, making anti-PCh IgM a more sensitive parameter in case of consumption. Preliminary experiments indeed indicate that anti-PCh IgM constitutes less than 1% of total IgM (N. Diaz Padilla, unpublished observation). Furthermore, we did not observe differences in anti-apo IgM or anti-PCh IgM, corrected for total IgM, on admission versus after 48 hours. In another study we observed in patients deceased after AMI that IgM binding to ischemic cardiomyocytes, appears in the infarcted myocardium approximately 24 hours after the occlusion of a coronary vessel as based on pathological criteria [13]. Therefore, we expected a further decrease of anti-PCh IgM in the patients with AMI during the first 48 hours after admission, but this was not observed. Further studies should reveal whether increased production of anti-PCh IgM has masked a decrease of levels due to localization in the infarcts.

Several studies have shown significant changes in plasma IgM and IgG levels upon tissue injury for examples in case of burns [32]. In contrast, we found no significant change of anti-apo IgM or anti-PCh IgM levels in patients with AMI during the first 48 hours. Though these data do not exclude that also AMI may induce an immunoglobulin response, such a response at least does not seem to occur within the observation period. Furthermore, similarity in levels on admission and after 48 hours, indicate that the analysis of levels on admission was not blurred by ongoing responses. Therefore, we decided to analyse whether patients with high levels on admission had a more intense inflammatory response than patients with lower levels of anti-apo or anti-PCh IgM.

Though patients with high levels of anti-apo IgM or anti-PCh IgM on admission had similar levels of IL6 and IL8 as patients with low levels (Table 2), their concentrations of CRP, sPLA2 and of complement activation products tended to increase. When the analysis was limited to the anti-PCh IgM specificity alone, sPLA2 concentrations were significantly higher in the group above median value. These data suggest that IgM with specificity for PCh is associated with the inflammatory response in patients with AMI.

Infarct size is determined by a number of parameters including localization of the occlusion, collateral circulation, treatment, and others [33;34]. Therefore, we did not expect to find an association between levels of anti-apo IgM or anti-PCh IgM and parameters of cardiac damage in this limited number of patients. Yet, patients with anti-PCh IgM on admission tended to have higher cumulative release of CK and LDH. Assessment of infarction size using electrographical score is only possible in case of a first infarction. Among patients with a first infarction, those who had higher anti-PCh IgM had a significantly higher Selvester score on admission than those with lower anti-PCh IgM. IgM is a known activator of complement and has been shown to bind apoptotic surfaces, in particular to phosphorylcholine [16] and activate complement [15]. Taken together, these observations would suggest that IgM antibodies particularly anti-PCh IgM may participate in the activation or amplification of the inflammation after AMI via the activation of complement.

Anti-PCh IgM can bind to apoptotic cells [16;28]. Competition experiments with soluble phosphorylcholine, however, indicate that not all IgM directed against apoptotic cells is against PCh [31]. Our results do not point to a role of anti-apoptotic cell IgM with other specificities in myocardial infarction. Yet it should be noted that a relationship between plasma levels of this IgM and inflammatory responses and infarct size only become clear when plasma levels are limiting. Thus, our data do not definitely rule out a role for IgM binding to apoptotic cells in local inflammatory reactions ensuing in the infarcted myocardium.

In conclusion, levels of anti-PCh IgM in patients with AMI are associated with a somewhat more intense inflammatory response and a larger infarct size. These data fit with the hypothesis that this IgM species by enhancing inflammatory damage to the heart during infarction, may contribute to infarction size.

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CHAPTER 7

Summarizing discussion

Ischemia-reperfusion injury (IRI) is an important pathophysiological event that occurs in many clinical conditions, ranging from surgery (cardiovascular bypass, abdominal aneurysm repair, transplantation), acute artery occlusion (stroke, gut ischemia, acute myocardial infarction (AMI)) to hemorrhagic shock. IRI may increase the morbidity and mortality of patients undergoing such events [1-3]. Complement activation is thought to be an important mediator in IRI, because complement inhibition and complement deficiency considerably attenuate irreversible injury [4-7]. However, the specific complement pathways involved remain unclear. All three complement pathways: the classical, the alternative, and the mannose-binding lectin (MBL)-dependent pathway may be involved in the development of IRI, depending on the model, the tissue, and the time course of inflammation [6,8-10]. This thesis focuses on the role of CRP and natural IgM Ab in tissue damage after ischemia and reperfusion. Ischemia leads to the exposition of "altered phospholipids" and neoantigens on the damaged tissue that can be recognized by C-reactive protein (CRP) or/and natural IgM antibody (natural IgM). The binding of both plasma proteins to ligands on the damaged tissue is followed by complement activation [11-15].

Complement activation by rat CRP

Because important biological functions of proteins are generally conserved among species, and because CRP has a very stable phylogenetic conservation of amino-acid sequence, subunit composition and binding specificity for phosphorylcholine (Pc), the role of CRP in mediating complement activation is likely to be similar in all animals [reviewed in 16].

To test this idea, we re-evaluated the capability of rat CRP to activate the rat complement system, as described in the Chapter 2. Complement activation was investigated in solid and fluid-phase systems. In the solid phase, rat and human CRP were fixed to ELISA plates and incubated with human and rat plasma. Dose-dependent binding of human and rat activated complement factors (C3b/c and C4b/c) were observed to human CRP and rat CRP. The generation of rat CRP-complement complexes (specific markers for CRP-dependent complement activation), after incubation of rat plasma with CRP ligands, i.e. *C-polysaccharide of pneumococci* (CPS) and lysophosphatidylcholine (Lyso-Pc), demonstrated this ability of rat CRP in the fluid phase. Furthermore, it was shown that complement activation by rat CRP occurs through the classical pathway, because it binds to C1q (both human and rat C1q). Next to these in vitro studies, we investigated whether CRP could activate complement in rat models of ischemia-reperfusion. Indeed, CRP-C3 complexes were detected in plasma in models for hind-limb and liver ischemia-reperfusion [17] and bacterial meningitis (data not shown). These findings together suggest that rat CRP can also activate autologous complement *in vivo*.

The discrepancy between our observations and previous findings that reported that rat CRP was unable to activate complement, is presumably a result of the use of different CRP ligands and methods to assess complement activation. Whereas we used a sensitive assay for the quantitation of CRP-complement complexes, in combination with other assays, and CPS and Lyso-Pc as ligands, de Beer *et al.* [18] used crossed immunoelectrophoresis, which is less sensitive than our ELISAs, and only CPS as CRP ligand.

A requirement for the activation of complement by CRP is that the pentraxin must be clustered into dimers or trimers, which is achieved when it is bound to suitable ligands, such as Pc in membranes of damaged cells [12,19-21]. Chapter 3 reports that although rat CRP levels increase upon estrogen replacement, this does not result in complement activation. Two

different doses of 17β-estradiol were administered to ovariectomized F-344 rats. The plasma CRP levels increased after estrogen administration. The fact that this increase did not result in complement activation in this model is consistent with the idea that suitable CRP ligands are also required.

Role of CRP and natural IgM in a rat model of intestinal IRI

There is accumulating evidence that complement activation by CRP and natural IgM Ab are important contributors to IRI. In mouse models of ischemia-reperfusion, a role of IgM in this process has been postulated [6,22]. In humans, both IgM and CRP play a role in complement activation after AMI [1,23,24]. In rats, CRP-mediated complement activation seems to be involved in the increase of infarct size in rats subjected to coronary artery ligation in a model of AMI [25]. The relative contribution of both proteins in the development of IRI in rats is still not fully elucidated.

Chapter 4 deals with the role of CRP, IgM and anti-Pc IgM in complement activation in a rat model of intestinal IRI. The effect of C1-inhibitor on inflammatory mediators was evaluated. Rats were subjected to 1 h of superior mesenteric artery occlusion and 3 h of reperfusion. Intestinal injury and myeloperoxidase activity were increased following IR compared to the sham animals. Ischemia and reperfusion results in significant deposition of CRP, IgM and complement on injured intestinal tissue. A better correlation was found between CRP/complement than IgM/complement deposition, which suggests that CRP could be the dominant complement activator in this model. It was supported by the positive correlation between the levels of C4b/c and CRP measured in intestinal homogenates. We showed that C1-Inh reduces C4b/c generation as measured in the homogenates. However, significant decrease of activated C3 deposition in the injured intestines was not observed

Taken together; these findings indicate that in animals with high CRP levels, as seen in rats, CRP and not natural IgM Ab is the dominant complement activator during ischemia and reperfusion. We suggest that therapeutic inhibition of the binding of CRP and IgM to damaged tissue may be a new anti-inflammatory therapy to decrease mortality rates in clinical conditions associated with IRI [26]. Recently, it has been shown, that neo epitopes expressed after ischemia and reperfusion in rats are not only recognized by CRP and IgM but also by MBL leading to the lectin pathway activation of the complement [27]. Further studies on the relative contribution of the classical vs. MBL pathway are needed to elucidate the role of both pathways.

Anti-Pc IgM in healthy donors and during acute phase response

Natural IgM Ab binds Pc (anti-Pc IgM) on microorganisms and on altered-self molecules, such as oxidized phospholipids. These Pc molecules are present in oxidized lipoproteins and in the outer leaflet of the plasma membrane of apoptotic and necrotic cells [28-30]. The binding of anti-Pc IgM to their ligands is followed by complement activation [31]. Because of these shared abilities with CRP (binding to Pc and complement activation), we propose that anti-Pc IgM could be, like CRP, a cardiovascular risk factor [12,32-34]. Recently, it has been shown that anti-Pc IgM blocks the progression of atherosclerosis by inhibiting the uptake of oxLDL by scavenger receptors on macrophages [35,36]. Therefore, based on the proposed anti-

atherosclerotic role of anti-Pc IgM, studies on the relationship between circulating levels of anti-Pc IgM and the risk of cardiovascular disease deserve further investigation.

We developed and validated an ELISA system for the quantitation of anti-Pc IgM Abs. As described in chapter 5, levels of anti-Pc IgM in healthy donors differed by more than 100-fold, but there was no relationship between these levels and age. Our data do not support the notion that anti-Pc IgM in humans is increased in atherosclerosis, since in that case, some relationship between plasma levels and age would be expected. Because no correlation between plasma CRP and anti-Pc IgM was found, we speculate that IgM and CRP constitute two independent mechanisms involved in the elimination of injured cells. Future studies on anti-Pc IgM should delineate its role in human diseases such as cardiovascular disease.

To test the hypothesis that anti-Pc IgM can bind, as CRP [12], to neoantigens expressed on necrotic or apoptotic cells, circulating levels of anti-Pc IgM were quantified in patients with skin cancers treated by isolated limb perfusion with tumor necrosis factor-α. Most of these patients exhibited massive death of both malignant cells and endothelial cells of the tumour vasculature [37]. Following this procedure, a significant decrease of circulating anti-Pc IgM relative to total IgM Ab was found. This decrease is in agreement with the notion that anti-Pc IgM can bind to phospholipids exposed on damaged cells.

The levels of circulating anti-Pc IgM were measured also in plasma samples from patients with AMI, as described in chapter 6. The measurement was done in samples obtained on admission (t0) and 48 hours thereafter (t48). In order to restore blood flow to the affected area, 70 % of the patients were treated at admission with thrombolytic agents and 20% with percutaneous transluminal coronary angioplasty. The relationship between anti-Pc IgM, inflammatory mediators and infarct size as assessed with electrocardiography was further analyzed.

The levels of anti-Pc IgM were significantly lower in patients with AMI (t0) than in healthy donors. This may suggest that in the patients circulating anti-Pc IgM is removed from the circulation and deposited in the infarcted myocardium tissue. At t48 the blood circulation was restored again. Because of this restored circulation, we expected to find more anti-Pc IgM deposition and therefore lower anti-Pc titers in the plasma. However, no difference in anti-Pc IgM levels between t0 and t48 was observed. Krijnen *et al*. [23] have shown that IgM Abs are deposited in ischemic infarcts, 24 h after the occlusion of a coronary artery. These investigators did not measure the IgM levels in plasma. In our experiments, a possible explanation for the absence of decreased anti-Pc IgM levels in plasma maybe attributed to be increased production of IgM, including anti-Pc IgM.

Nevertheless, levels of anti-Pc IgM on admission correlated with the levels of the inflammatory mediator, secretory phospholipase A_2 ($sPLA_2$) and with the infarct size. IgM is a known activator of complement after binding to apoptotic surfaces like Pc. Taken together, these observations suggest that anti-Pc IgM participate in amplification of inflammation after AMI via activation of complement, and in that way increase the cardiac damage.

Concluding remarks

This thesis describes that CRP in rats is able to activate the classical pathway of the complement system. This means that rats are suitable animals to study the CRP-mediated complement activation after tissue damage. The relative contributions of CRP and IgM to activation of the complement system varies between species. In a rat model of intestinal ischemia-reperfusion, CRP (basal level about 500 mg/L) was shown to be the dominant

complement activator. The administration of anti-Pc IgM Abs in rat models of ischemia-reperfusion may be used to elucidate its potential impact. The decrease in plasma anti-Pc IgM levels found under conditions of excessive cell death, and the increased production of pro-inflammatory mediators, is in agreement with the notion that these Abs bind to apoptotic and necrotic cells. Thus, anti-Pc IgM amplifies inflammation and likely participates in the clearance of cell debris. The correlation found between levels of anti-Pc IgM, $sPLA_2$ and infarct size supports the notion that these Abs contribute to cell damage. Anti-Pc IgM levels were measured in healthy donors to study the variability in the population. Our data do not support the notion that these Abs are related with the process of atherogenesis, because no correlation between level and age was found. Further prospective studies on anti-Pc IgM in the healthy population should be performed to delineate their role in atherogenesis.

All together, CRP as well as anti-Pc IgM can be involved in processes following tissue damage. Clearing of apoptotic cells is an example of this involvement. Binding of CRP and IgM to damaged tissue can lead to an increased inflammation. Development of strategies to prevent binding of CRP and/or IgM can be useful to prevent further activation of effector mechanisms.

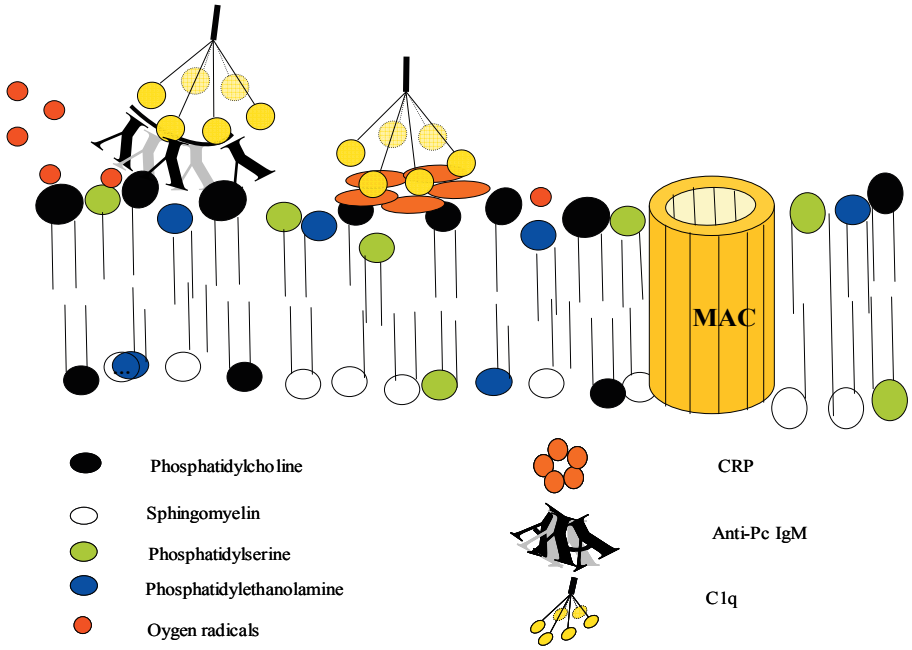

Diagram illustrating the proposed role of CRP, anti-Pc IgM and complement mediating ischemia and reperfusion injury on a cell membrane. *The flip-flop of the membrane, oxidation of the phospholipids by oxygen radicals and hydrolysis by phospholipases A_2 (not shown) after an ischemic insult generate binding sites, i.e. Pc, on the membranes. CRP and IgM bind to Pc. C1q binding to CRP and IgM triggers the complement cascade. Because damaged tissues lack the regulatory molecules that in normal tissues prevent excessive complement activation, complement might be completely activated up to the formation of the membrane attack complex (MAC). MAC leads to pore formation and the cell are irreversibly damaged.*

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Samenvatting

Ischemie-reperfusie-schade (IRS) is een belangrijk pathofysiologisch verschijnsel dat optreedt bij vele klinische condities, zoals chirurgische interventies (bypass-operatie, transplantatie), acute arteriële occlusie (darmischemie, acuut myocardinfarct) en hypovolemische shock. IRS kan de morbiditeit en de mortaliteit bij patiënten met deze aandoeningen verhogen. Vermoedelijk speelt complement activatie een belangrijke rol bij IRS, omdat remming van de complementactivatie en ook complementdeficiëntie een aanzienlijke afname van de irreversibele schade geeft. De specifieke mechanismen zijn echter nog onduidelijk. Er zijn drie complement activatie-routes bekend, te weten: de klassieke-, de alternatieve- en de mannose-bindend lectine (MBL)-afhankelijke route. Afhankelijk van de aandoening, het weefsel en de duur van de ontsteking kunnen alle drie activatie routes een rol spelen in het ontstaan van IRS. Dit proefschrift richt zich op de rol van de binding van CRP en natuurlijke IgM antistoffen aan beschadigd weefsel, in het bijzonder na ischemie en reperfusie. Ischemie leidt tot blootstelling van "veranderde fosfolipiden" en neoantigenen op cellen in het beschadigde weefsel, en deze kunnen vervolgens herkend worden door de plasma-eiwitten C-reactief protein (CRP) en/of door natuurlijke IgM antistoffen. De binding van beide plasma-eiwitten aan het beschadigde weefsel wordt gevolgd door complementactivatie.

Complementactivatie door ratten-CRP

Eiwitten met essentiële biologische functies zijn meestal geconserveerd tussen verschillende diersoorten. CRP is een sterk geconserveerd eiwit op basis van aminozuur-sequentie, subunitsamenstelling en binding aan phosphorylcholine (Pc). Dit suggereert dat CRP in verschillende zoogdieren een vergelijkbare rol speelt in complementactivatie.

Hierom hebben we opnieuw het vermogen van ratten-CRP onderzocht om het complementsysteem te activeren, zoals beschreven in hoofdstuk 2. Complementactivatie werd onderzocht in vaste-fase en vloeibare-fase systemen. In vaste-fase werd ratten-en humaan CRP gefixeerd op ELISA platen en geïncubeerd met ratten-en humaan serum. Dosis-afhankelijke binding van geactiveerde ratten-en humane complement-eiwitten C3b/c en C4b/c aan ratten-en humaan CRP werd vastgesteld. In de vloeibare-fase opzet werd CRP geïncubeerd met zijn liganden C-polysacharide van pneumococci (CPS) en lysophosphatidylcholine (Lyso-Pc). De vorming van CRP-complement complexen in dit systeem demonstreerde CRP-afhankelijke complementactivatie. Bovendien werd vastgesteld dat complementactivatie door ratten-CRP verliep via de klassieke route omdat het C1q bindt, en dat ratten-CRP zowel ratten-als humaan C1q kan binden. Naast deze *in vitro* studies hebben we in een rattenmodel van IRS bekeken of CRP het complementsysteem kan activeren. In die studie hebben we aan kunnen tonen dat er CRP-C3 complexen gevormd worden tijdens in ischemie en reperfusie van de achterpoot en van de lever, en in experimentele bacteriële meningitis. Deze gegevens zijn een sterke indicatie dat autologe complementactivatie door ratten-CRP *in vivo* optreedt.

Een eerdere studie toonde geen complementactivatie aan door ratten-CRP. Deze eerdere studie gebruikte echter alleen CPS als ligand en gebruikte bovendien de minder gevoelige 'crossed immunoelectrophoresis' als meetmethode. Dit kan het verschil met onze studie verklaren.

Een voorwaarde voor de activatie van complement door CRP is de aggregatie van CRP tot di- of trimeren. CRP aggregatie kan bewerkstelligd worden door binding aan geschikte liganden, zoals Pc in beschadigd weefsel. Hoofdstuk 3 beschrijft de invloed van oestrogenen op CRP-gemediëerde complementactivatie. Twee verschillende doseringen van 17β-estradiol capsules werden toegediend aan F-344 ratten na ovariëctomie. Het plasma-CRP niveau steeg na oestrogeentoediening, maar er werd geen complementactivatie gedetecteerd. Dit geeft aan dat stijging van het CRP gehalte niet tot complementactivatie leidt als niet ook de geschikte liganden gelijktijdig gegenereerd worden.

Rol van CRP en natuurlijk IgM in een rattenmodel voor intestinale IRS

Er zijn vele aanwijzingen dat complementactivatie door CRP en natuurlijke IgM antistoffen in belangrijke mate bijdragen aan IRS. In muizenmodellen van IRS is een rol voor IgM gepostuleerd. In de mens is gevonden dat zowel IgM als CRP een rol spelen bij complementactivatie na acuut myocardinfarct. In een rattenmodel voor acuut myocardinfarct lijkt CRP-gemediëerde activatie gecorreleerd aan de grootte van het infarct na kransslagaderligatie. Echter, de relatieve bijdrage van beide eiwitten in de ontwikkeling van IRS in ratten is nog steeds niet volledig opgehelderd.

Hoofdstuk 4 gaat in op de rol van CRP, IgM en anti-Pc IgM in complementactivatie in een rattenmodel voor intestinale IRS. Het effect van C1 esteraseremmer op inflammatoire mediatoren wordt hierin beschreven. Ratten werden onderworpen aan een acute afsluiting van de arteria mesenterica superior gedurende 1 uur, gevolgd door een 3 uur durende reperfusie.

Intestinale schade en myeloperoxidaseactiviteit waren toegenomen gedurende ischemie en reperfusie, vergeleken met controledieren. Ischemie en reperfusie resulteerde in een significante depositie van CRP, IgM en complement op beschadigd intestinaal weefsel. De correlatie die gevonden werd tussen CRP en complement was sterker dan die tussen IgM en complement. Dat suggereert dat CRP mogelijk de dominante complement-activator is in dit model. Dit werd ondersteund door de positieve correlatie tussen C4b/c en CRP, gemeten in intestinale homogenaten. Wij toonden aan dat C1- esteraseremmer de C4b/c productie in intestinaal tissue blokkeert. We konden echter geen significante afname van C3 depositie in het beschadigde weefsel aantonen.

Alles tezamen suggeren deze resultaten dat in dieren met hoge CRP titers, zoals ratten, CRP de dominante complementactivator is gedurende IRS en niet natuurlijke IgM antistoffen. Therapeutische remming van de binding van CRP en IgM aan beschadigd weefsel kan een nieuwe anti-inflammatoire therapie worden. De morbiditeit en de mortaliteit in klinische condities geassocieerd met IRS kunnen hierdoor afnemen. Onlangs is in ratten aangetoond dat neo-epitopen die tot expressie komen na ischemie en reperfusie niet alleen worden herkend door CRP en IgM, maar ook door MBL, hetgeen leidt tot complementactivatie via de ‘lectineroute’. Verdere studies aan de relatieve bijdrage van de MBL-route ten opzichte van de klassieke route zijn nodig om het belang van beide op te helderen.

Anti-Pc IgM in gezonde donoren en gedurende de acute-fase respons

Natuurlijke IgM antistoffen kunnen binden aan Pc op microorganismen en op veranderde eigen-antigenen zoals geoxideerde fosfolipiden. Deze worden gevonden op lipoproteinen en in het celmembraan van apoptotische en necrotische cellen. De binding van anti-Pc IgM aan

zijn liganden wordt, net als bij CRP, gevolgd door complementactivatie. Recentelijk is vastgesteld dat anti-Pc IgM athereosclerose afremt. Door binding van anti-Pc IgM aan OxLDL wordt OxLDL niet meer herkend door de scavenger receptor op macrofagen en daardoor niet opgenomen. Omdat CRP en anti-Pc IgM beide binden aan Pc en beide complement kunnen activeren, stellen we voor om anti-Pc IgM, net als CRP, als risico factor voor cardiovasculaire aandoeningen te beschouwen. Echter, gezien de mogelijke anti-arthereosclerotische rol van anti-Pc IgM, is meer onderzoek nodig naar de relatie tussen circulerende anti-Pc IgM titers en het risico van cardiovasculaire ziekte .

Er is een ELISA voor de kwantificering van anti-Pc IgM ontwikkeld en gevalideerd. Dit is beschreven in hoofdstuk 5. De anti-Pc IgM titers in gezonde donoren varieerde met meer dan een factor 100, maar was er geen relatie tussen titer en leeftijd. Onze data ondersteunen dus niet de gedachte dat humaan anti-Pc IgM verhoogd is in arthereosclerosis, omdat dan enige relatie met leeftijd verwacht zou worden. Er werd ook geen correlatie gevonden tussen plasma-CRP en anti-Pc IgM titers. Dit suggereert dat anti-Pc IgM en CRP twee aparte mechanismen vormen bij de eliminatie van beschadigde cellen. Het wordt vaker gezien dat essentiële biologische mechanismen op meer dan één manier gereguleerd worden, om het effect van ontsporingen te verkleinen. Vervolgstudies kunnen meer licht werpen op de rol van anti-Pc IgM antilistoffen in cardiovasculaire ziekten.

Om de hypothese te toetsen dat anti-Pc IgM, net als CRP kan binden aan neo-antigenen die tot expressie komen op necrotische of apoptotische cellen, hebben we de anti-Pc IgM titers gemeten in patiënten met huidkanker die behandeld werden met tumor necrosis factor-α in regionale perfusie. In de meeste van deze patiënten werd massale sterfte gemeten van zowel tumorcellen als endotheelcellen van de tumorvasculatuur. Tijdens deze procedure werd een significante afname van anti-Pc IgM gemeten, gerelateerd aan de totaal IgM titers. Deze afname kan verklaard worden doordat anti-Pc IgM bindt aan de beschadigde cellen en zo uit de circulatie verdwijnt.

De titers van circulerend anti-Pc IgM werden ook bepaald in plasma van patiënten met acuut myocardinfarct, zoals beschreven in hoofdstuk 6. De bepaling werd uitgevoerd in monsters verkregen bij opname in het ziekenhuis (t0) en 48 uur daar (t48). Om de bloedcirculatie in het aangedane weefsel te herstellen werden 70% van de patiënten behandeldeld met een thrombolyticum (stolsel-oplossend middel) en 20% werd behandeld met directe angioplastiek (PTCA of dotterprocedure). De correlatie werd bepaald tussen anti-Pc IgM, inflammatoire mediatoren en de grootte van het infarct, gemeten met elektrocardiografie. De anti-Pc IgM titers in acuut myocardinfarct patiënten (t0) waren significant lager dan in de gezonde donoren. Dit suggereert dat de plasma titers van het anti-Pc IgM lager worden door depositie van anti-Pc IgM in het aangedane weefsel. Op t48 is de bloedcirculatie weer hersteld en hierdoor zou nog meer depositie anti-Pc IgM kunnen optreden. We vonden echter geen additionele afname van anti-Pc IgM na 48 uur. Krijnen *et al.* hebben IgM-depositie gevonden in hartweefsel op 24 uur na afsluiting van een kransslagader. Ze hebben echter geen IgM gemeten in plasma. Een mogelijke verklaring voor het uitblijven van een verdere daling van anti-Pc IgM na herstel van de circulatie in onze experimenten is een verhoogde productie van anti-Pc IgM. Niettemin, anti-Pc IgM-titers bij ziekenhuisopname correleren met de ontstekings-mediator secretoroir fosfolipase A_2 ($sPLA_2$) en met de grootte van het infarct. IgM is een bekende activator van het complementsysteem, met name door binding aan apoptotische oppervlakken die Pc epitopen bevatten.

Samengevat kan gesteld worden dat zowel CRP als anti-Pc IgM antistoffen betrokken kunnen zijn bij processen waarbij de integriteit van weefsel aangedaan is. In het geval van klaring van apoptotische cellen bijvoorbeeld, kunnen CRP en IgM antistoffen bijdragen aan

de verwijdering van dit soort materiaal. Wanneer de binding van CRP en IgM plaats vindt aan beschadigd weefsel kan dit leiden tot verdere versterking van ontsteking. In dit soort gevallen zou men graag strategieën willen ontwikkelen om de binding van CRP en IgM te blokkeren of de activatie van effectormechanismen te reduceren.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 27 november 1966 te Havana, Cuba. Het doctoraal examen geneeskunde aan de Medische Universiteit van Havana werd in 1990 cum laude afgesloten. Aansluitend volgde zij de opleiding Klinische Chemie (medische specialisatie) aan de afdeling Biochemie van dezelfde Universiteit tot 1995, waar zij de titel specialist ontving. Vanaf 1990-1998 heeft ze gewerkt als onderzoeker in "Centro de Ingieneria Genética y Biotecnología" (CIGB) in Havana. In aansluiting hierop heeft zij in 1998 als onderzoeker gewerkt aan de glycosilering van recombinante eiwitten, o.a. op de afdeling Endocrinologie van het "National Institute of Biological Standards and Control (NIBSC)", UK (o.l.v. B. Rafferty). In 1998 is zij naar Nederland verhuisd. Na een periode van inburgeren heeft zij stage gelopen op de afdeling Medische Chemie, op de Faculteit der Geneeskunde van Vrije Universiteit onder begeleiding van dr. I. van Die. Het onderzoek werd verricht naar "Differential galactosylation of neuronal and haematopoietic signal regulatory protein-alpha determines its cellular binding specificity". In juni 2000 is zij in dienst getreden als onderzoeker in opleiding op de afdeling Immunopathologie van Sanquin Research. Het onderzoek naar de rol van plasma eiwitten (CRP en IgM) in ischemie-reperfusie schade werd verricht onder leiding van Prof. dr. C.E. Hack en heeft uiteindelijk geresulteerd in dit proefschrift onder leiding van Prof. dr. M.R. Daha. Vanaf januari 2005 loopt ze co-schappen bij het Universitair Medisch Centrum Utrecht (UMC).

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