Complement activation and disease: protective effects of hyperbilirubinaemia

Cecilia L. BASIGLIO*, Sandra M. ARRIAGA†, Fabián PELUSA†, Adriana M. ALMARÁ‡‡, Jaime KAPITULNIK§∥ and Aldo D. MOTTINO*

*Institute of Experimental Physiology (CONICET), National University of Rosario, S2002LRL-Rosario, Argentina, †Department of Clinical Biochemistry, National University of Rosario, S2002LRL-Rosario, Argentina, ‡CIURN, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, S2002LRL-Rosario, Argentina, §Department of Pharmacology, School of Pharmacy, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem 91120, Israel, and ∥David R. Bloom Center for Pharmacy and Dr Adolph and Klara Brettler Center for Research in Molecular Pharmacology and Therapeutics, School of Pharmacy, Hebrew University of Jerusalem, Jerusalem 91120, Israel

ABSTRACT

Complement, an important effector mechanism of the immune system, is an enzymatic cascade of approx. 30 serum proteins leading to the amplification of a specific humoral response. It can be activated through the classical or alternative pathways, or through the mannose-binding lectin pathway. The activation of the classical pathway is initiated by the binding of the C1 component to antigen-bound antibodies, known as immunocomplexes. C1 is a complex of one molecule of C1q, two molecules of C1r and two molecules of C1s. C1q contains three copies of a Y-shaped fundamental unit with globular heads included in its structure, which play a major role in the interaction with the Fc portion of immunoglobulins. Deficient or exacerbated activation of the complement system leads to diseases of variable severity, and pharmacological inhibition of the complement system is considered as a therapeutic strategy to ameliorate the inflammatory effects of exacerbated complement activation. Bilirubin is a product of haem degradation by the concerted action of haem oxygenase, which converts haem into biliverdin, and biliverdin reductase, which reduces biliverdin to UCB (unconjugated bilirubin). UCB exerts both cytoprotective and cytotoxic effects in a variety of tissues and cells, acting either as an antioxidant at low concentrations or as an oxidant at high concentrations. In the present review, we describe in detail the anti-complement properties of bilirubin, occurring at levels above the UCB concentrations found in normal human serum, as a beneficial effect of potential clinical relevance. We provide evidence that UCB interferes with the interaction between C1q and immunoglobulins, thus inhibiting the initial step in the activation of complement through the classical pathway. A molecular model is proposed for the interaction between UCB and C1q.

COMPLEMENT SYSTEM

The complement system is an enzymatic cascade of approx. 30 serum proteins leading to the amplification of a specific humoral response. Activation and fixation of complement to micro-organisms constitute an important effector mechanism of the immune system that facilitates the elimination of antigen and generates an inflammatory response [1].

Key words: complement, cytoprotection, hyperbilirubinaemia, immune system, liver disease, oxidative stress, unconjugated bilirubin.

Abbreviations: BOX, bilirubin oxidized product; BV, biliverdin; CP, classical pathway; CR, complement receptor; CYP1A1, cytochrome P450 1A1; HBV, hepatitis B virus; HCV, hepatitis C virus; MAC, membrane-attack complex; MBL, mannose-binding lectin; RNS, reactive nitrogen species; ROS, reactive oxygen species; UCB, unconjugated bilirubin; Ugt1a1/UGT1A1, UDP glucuronosyltransferase 1A1.

Correspondence: Dr Aldo D. Mottino (email amottino@unr.edu.ar).
The main physiological effects of activation and fixation of the complement system include: (i) lysis of target cells or micro-organisms; (ii) opsonization of antigens or immunocomplexes, which optimizes phagocytosis and elimination of immunocomplexes; (iii) improvement of phagocyte chemotaxis and the control of the inflammatory response by diffusible products of activated complement, the anaphilotoxins C3a and C5a; (iv) amplification of the specific humoral response; and (v) neutralization of certain viruses [1].

Innate responses frequently involve complement, acute-phase proteins and cytokines. The early events of complement activation, which are based on an enzymatic amplifying cascade comparable with that seen in blood clotting, can be triggered by one of three pathways: the CP (classical pathway), the alternative pathway and the MBL (mannose-binding lectin) pathway (Figure 1) [2]. The CP is activated by antigen–antibody complexes and is initiated by the binding of the C1 component of complement to the Fc fragment of the antibody molecule. The alternative pathway is activated directly by microbial cell walls. The MBL pathway resembles the CP, but is initiated in the absence of antibodies by the interaction of microbial carbohydrates with mannose-binding proteins in the plasma [3]. Irrespective of the source of activation, the final outcome is the generation of a number of immunologically active substances. A proteolytic cleavage fragment of the complement component C3, the C3b molecule, becomes deposited on the surface of micro-organisms. This event enhances phagocytosis of the microbe, as a consequence of phagocytic cells having cell-surface receptors for C3b. The complement fragments C3a, C4a and C5a release inflammatory mediators from mast cells. C5a also acts as a powerful neutrophil chemoattractant. All three activation routes converge at the level of C3 to form the C5 convertase. The sequential binding of the complement components C5b, C6, C7, C8 and C9 form the MAC (membrane-attack complex), which perforates cell membranes and thereby leads to the death of the target cell (reviewed in [4]). A schematic representation of the CP and final MAC structure is shown in Figure 2.

For every stage of activation of the CP there are regulatory proteins which bring about rapid destruction of the activated factor, thereby preventing complete consumption of plasma C4 and C2 in the fluid phase. This is the reason for the evolutionary creation of various regulatory strategies which prevent host damage. Rapid inactivation of C1 to prevent uncontrollable activation of complement is brought about by the non-enzymic protein C1 inhibitor. C3 convertase is eliminated in two phases. First, C2a dissociates spontaneously from C4b, a process which is promoted by the C4-binding protein and DAF (decay-accelerating factor; CD55), which is found within the membrane of red blood cells and many different cell types. In the second phase, residual bound C4b is degraded further into C4c, which dissociates from the target membrane, leaving only C4d attached to this membrane. This degradation is brought about by the enzyme factor I, aided by C4-binding protein and the C3 receptor CR1. Factor I also cleaves bound C3b. Once C3 is inactivated it can no longer bind C5, hence further production of MAC is prevented. Plasma protein S and membrane protein CD59 also avoid reactive lysis by MAC on self-membranes. Finally, various components of the activated complement system are unstable intermediates in solution and are rapidly degraded when they are not in close proximity to the target cell ([5], and reviewed in [6]).

The activation of the CP is initiated by the binding of C1 to antigen-bound antibodies. C1 is a complex of one molecule of C1q, two molecules of C1r and two molecules of C1s (Figure 3), which is stabilized by Ca$^{2+}$ ions. C1q is a 460 kDa hexameric protein comprising six heterotrimeric collagen-like triple-helical fibres, each containing a C-terminal globular region that supports most, if not all, of the C1 recognition activities (reviewed in [7,8]). Thus the image of the whole C1q molecule resembles that of a ‘tulip bunch’ with 18 polypeptide chains and six globular heads. C1q has been shown to be capable of recognizing over 50 structurally diverse ligands. Some of these ligands (e.g. aggregated IgG and IgM) bind to the globular domains of C1q, whereas other ligands, such as C-reactive protein, have been shown to

![Diagram of complement pathways](image-url)
Anti-complement properties of bilirubin

Figure 2  The CP of complement activation
The activation of the CP is initiated by the binding of C1 to antigen-bound IgG or IgM antibodies, which results in activated C1. The next step in the cascade is the catalytic cleavage of C4 and C2 by the activated C1, thus rendering C4b2a, or C3 convertase of the CP, which in turn cleaves C3 to give C4b2a3b or C5 convertase of the CP. This enzyme acts on C5, resulting in the generation of C5b. The sequential binding of C6, C7, C8 and C9 gives rise to C5b-9 or MAC. The schematic structure of MAC is shown at the end of this cascade.

Figure 3  Structure of the C1 component
The C1 component is found in fresh serum as a pentamolecular complex formed by one molecule of C1q, two molecules of C1r and two molecules of C1s, which are kept together in the presence of Ca^{2+} ions.

BIOLOGICAL IMPLICATIONS OF THE ACTIVATION OF THE COMPLEMENT SYSTEM

The three main physiological activities of the complement system are as outlined below.

Host defence against infection
The opsonization of antigens and immunocomplexes occurs through the covalent binding of C3 and C4 fragments. The anaphylatoxins C3a and C5a and their receptors on leucocyte surfaces are responsible for
chemotaxis and activation of white blood cells. Finally, the assembly of MAC results in the lysis of viruses, bacteria and cells ([11], and reviewed in [12]).

Interface between innate and adaptive immunity
C3b and C4b, bound to immune complexes and antigens, increase the antibody responses, as is the case for C3 receptors on B-cells and antigen-presenting cells. Furthermore, ‘immunological memory’ is enhanced by C3b and C4b fragments bound to immunocomplexes and to antigens, as well as by C3 receptors on follicular dendritic cells (reviewed in [12]).

Disposal of waste
C1q and covalently bound fragments of C3 and C4 are responsible for the clearance of immunocomplexes and apoptotic cells (reviewed in [12]).

DISEASES ASSOCIATED WITH ACTIVATION OF THE COMPLEMENT SYSTEM
The complement system has critical immunoprotective and immunoregulatory functions, but complement activation can also lead to severe disturbances.

Asthma
It has been proposed that the complement system plays an important role in the pathogenesis of asthma. Humbles and co-workers [13] and Bautsch and co-workers [14] have shown that C3a-receptor-deficient mice and guinea-pigs are protected against bronchoconstriction and airway hyper-responsiveness produced by allergen challenge. Krug et al. [15] suggested that anaphylatoxins C3a and C5a contribute to the pathogenesis of asthma, as both of these complement factors are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. Baelder et al. [16] proposed an important and exclusive role for C5a receptor signalling in the development of airway hyper-responsiveness during pulmonary allergen challenge, whereas both anaphylatoxins contribute to airway inflammation and IL-4 (interleukin-4) production.

Acquired haemolytic anaemia
Immune activation is a major cause of acquired haemolytic anaemia. Physiopathologically, the term haemolysis refers to a shortened survival of erythrocytes in blood. Aetiologically, haemolysis is caused by various factors that injure the erythrocyte, thus leading to its accelerated disappearance from blood [17]. Immune-mediated haemolysis is caused by the binding of IgG or IgM antibodies to the erythrocyte membrane surface. IgG antibodies facilitate the clearance of erythrocytes by the phagocytic mononuclear system. This effect is mediated by receptors located on the macrophage membrane, which fix the Fc region of the IgG. In contrast, the activity of IgM antibodies depends on complement activation, as macrophages lack specific receptors for their Fc fragment. Immune-mediated haemolysis in vivo can be classified into two basic types: (i) intravascular haemolysis, which occurs when erythrocytes are destroyed in the circulatory system due to complete activation of the complement system on the erythrocyte membrane, and (ii) extravascular haemolysis, in which erythrocytes are destroyed by the phagocytic mononuclear system. Erythrocytes coated with IgG1 or IgG3 and/or C3b are destroyed after binding to Fcγ receptors and/or CR1 in the phagocytic mononuclear cells ([17] and reviewed in [18]).

Alzheimer’s disease
Alzheimer’s disease is a neurodegenerative disease resulting in progressive cognitive decline. Amyloid plaque deposits, consisting specifically of β-amyloid peptides that have formed fibrils displaying a β-pleated sheet conformation, are associated with activated microglia and astrocytes, and co-localize with C1q and other complement activation products, and appear at the time of cognitive decline in Alzheimer’s disease. APP (amyloid precursor protein) transgenic mouse models of Alzheimer’s disease, which lack the ability to activate the CP, have a reduced neuropathology compared with wild-type mice, consistent with the hypothesis that complement activation and the resultant inflammation may play a role in the pathogenesis of Alzheimer’s disease [19]. Plaque- and cell-associated C3 immunoreactivity is observed in both animal models and, surprisingly, is increased in transgenic mice, thus providing evidence for activation of the alternative pathway [20]. The unexpected increase in C3 levels in transgenic mice, in conjunction with a decrease in neuropathology, provides support for the hypothesis that complement can mediate protective as well as detrimental events in this disease [19].

DEFICIENCY IN COMPLEMENT ACTIVATION CASCADE COMPONENTS AND REGULATORY PROTEINS AS A CAUSE OF DISEASE

Autoimmune diseases
Purified C1q and MBL bind to apoptotic cells and facilitate their phagocytosis by macrophages [21,22]. Deficiencies in these early components of the complement activation cascade result in impaired clearance/removal of apoptotic cells by macrophages upon autoimmune activation. It has been shown that inherited C1q deficiency is associated with an elevated risk for the development of systemic lupus erythematosus (reviewed in [23]).
**Hereditary angioedema**

Deficiency in complement regulatory proteins is also involved in hereditary angioedema. C1 inhibitor deficiency is the genetic defect underlying this pathology. Patients with hereditary angioedema suffer from recurrent episodes that may result in death when it affects the larynx, in severe abdominal pain when it affects the gastrointestinal mucosa and in disfigurement when it affects the skin [24].

**Paroxysmal nocturnal haemoglobinuria**

In the rare haemolytic disease paroxysmal nocturnal haemoglobinuria, somatic mutations result in a deficiency in glycosylphosphatidylinositol-linked surface proteins of haemopoietic stem cells, including the terminal complement inhibitor CD59. In a dysfunctional bone marrow background, these mutated progenitor blood cells expand and populate the periphery. Deficiency in CD59 in paroxysmal nocturnal haemoglobinuria red blood cells results in chronic complement-mediated intravascular haemolysis, a process central to the morbidity and mortality in this disease [25].

**Myocardial ischaemia/reperfusion injury**

Myocardial ischaemia/reperfusion injury processes are associated with complement activation. Ischaemic tissues lose complement regulatory proteins and, consequently, their anti-complement self-protection, thus activating the alternative pathway. In addition, the CP can be activated by antibodies against antigens that are released by the necrotic tissue, such as the cardiac mitochondrial particle cardiolipin, or by the fibrinolytic system. Accordingly, the complement proteins C1q, C3, C4 and C5, and MAC components are present in ischaemic areas [26,27].

**LIVER DISEASES ASSOCIATED WITH COMPLEMENT ACTIVATION**

**Liver fibrosis**

C5 and C5a receptor have been shown to play a critical role in the pathogenesis of liver fibrosis. Immunocytochemistry and confocal laser scanning microscopy analyses showed the expression of high levels of C5a receptor 1 on hepatic stellate cells during trans-differentiation to myofibroblasts in culture, which increased significantly in dead cells [28]. Stimulation of C5a receptor 1 by C5a can up-regulate fibronectin expression and induce prostanooid release in hepatic stellate cells [29,30].

**Viral hepatitis**

More than half a billion people worldwide are chronically infected with HBV (hepatitis B virus) or HCV (hepatitis C virus), which are a leading cause of cytolyis, fibrosis and cirrhosis. The mechanisms responsible for virus persistence and disease pathogenesis remain poorly understood, although an interaction of hepatitis viruses with the host immune system is probably involved (reviewed in [31]). Cold activation of complement was reported in patients with chronic HCV infection, which contributed to HCV-associated liver damage and was useful in monitoring a response to interferon therapy in these patients [32,33]. Immunohistochemistry showed that MAC was present in hepatocytes surrounding necrotic areas in patients with fulminant and acute hepatitis [34], supporting further the concept that the complement system is activated and involved in the pathogenesis of HCV-associated liver disease.

Genetic polymorphisms are one of the most important factors that determine plasma levels of MBL. Individuals possessing variant alleles of MBL have low plasma levels of this protein. MBL has a dose-dependent correlation with cirrhosis and hepatocellular carcinoma in progressed HBV carriers [35,36], who probably become viral-persistent after HBV infection [37]. Moreover, it was also demonstrated that MBL binds hepatitis B surface antigen in vitro in a dose- and calcium-dependent, and mannan-inhibited manner, and that this binding also enhanced C4 deposition [36]. Finally, chronic HBV and HCV infection resulted in a decrease in serum MBL levels, which was probably due to impaired hepatic production of this protein [38]. Taken together, these findings suggest that patients with variant alleles of MBL have low levels of this protein, which are associated with progression of liver disease and viral persistence after HBV infection (reviewed in [39]).

**Liver ischaemia/reperfusion injury**

Liver ischaemia/reperfusion injury occurs during liver surgery and transplantation, and is triggered by a complex inflammatory response following temporary deprivation of blood supply. Two distinct phases of hepatic ischaemia/reperfusion injury have been identified. The initial phase involves Kupffer cell activation and production of oxidative stress. The later phase is characterized by massive neutrophil infiltration (reviewed in [40]). The involvement of complement activation in liver ischaemia/reperfusion injury in a rat model was first reported by Jaeschke et al. [41]. They demonstrated that depletion of serum C, by treatment with cobra venom factor before ischaemia, prevented Kupffer-cell-induced oxidative stress, accumulation of polymorphonuclear leucocytes in the liver and hepatic injury caused by ischaemia/reperfusion. Furthermore, treatment with a C5a receptor antagonist reduced total hepatic ischaemia/reperfusion-induced mortality and ameliorated the partial ischaemia/reperfusion-induced liver injury and accumulation of polymorphonuclear leucocytes in the liver (reviewed in [42]). Deposition of MAC was found to be elevated in the post-operative specimens of patients with liver transplantation, and correlated positively with the number of leucocytes and platelets accumulating within the graft, and with an increase in...
post-operative serum levels of aspartate aminotransferase [43]. This suggests that complement activation may contribute to rejection after liver transplantation [44]. Moreover, complement activation may play an important role in the rejection of hepatic xenotransplants [45].

**THERAPEUTIC APPLICATION OF COMPLEMENT SYSTEM INACTIVATION**

Understanding the mechanisms which down-regulate the action of complement could contribute to the development of therapeutic strategies to ameliorate the inflammatory effects in diseases that involve complement activation. It is worth noting that some drugs, through their anti-complement action, attenuate symptoms in diseases that involve an inflammatory component. The root extract from *Glycyrrhiza glabra* (regaliz) is known for its anti-inflammatory and anti-allergic properties, which are attributed to one of its main components, β-glycyrrhetinic acid. Kroes et al. [46] demonstrated that this steroid component is able to inhibit the CP. This property was found to be strictly dependent on the conformational structure of β-glycyrrhetinic acid, as its α-isomer did not exhibit anti-complement activity. A mechanistic study showed that β-glycyrrhetinic acid acts through its modulatory properties on the C2 component [46].

Therapeutic inhibition of the complement cascade at the C5 level using specific antibodies might impair the formation of the potent anaphilotoxin and chemotactic peptide C5a, thus attenuating the inflammatory reactions mediated by complement (reviewed in [47]). In this regard, it was demonstrated in a murine model of systemic lupus erythematosus that therapy with monoclonal anti-C5 antibody resulted in a significant decrease in the progression of lupic glomerulonephritis and in a marked increase in animal survival [48]. A humanized monoclonal antibody directed against C5, eculizumab, blocks the pro-inflammatory and cytolytic effects of terminal complement activation. The recent approval of eculizumab as a first-in-class complement inhibitor for the treatment of paroxysmal nocturnal haemoglobinuria validates the concept of complement inhibition as an effective therapy, and provides the rationale for the investigation of other conditions in which complement plays a pathogenetic role [25].

Cobra venom factor, the complement-activating protein in cobra venom, is a structural and functional analogue of C3. In serum, cobra venom factor forms a physicochemically stable and regulation-resistant C3/C5 convertase that continuously activates C3 and C5, leading ultimately to depletion of serum complement. Complement depletion is an attractive concept for pharmacological intervention in diseases where complement activation is part of the pathogenic mechanism. The structural homology of cobra venom factor and C3 has been exploited by creating hybrid proteins in which short portions of C3 sequence have been exchanged with corresponding portions of cobra venom factor that introduce into human C3 the desired ability of forming a stable convertase. These human C3 derivatives are ‘humanized cobra venom factor’ proteins that provide an attractive biopharmaceutical tool for therapeutic complement depletion [49,50].

Despite antibiotic treatment, pneumococcal meningitis continues to be associated with significant morbidity and mortality. C1 inhibitor treatment was associated with reduced clinical illness, a less-pronounced inflammatory infiltrate around the meninges, and lower brain levels of pro-inflammatory cytokines and chemokines. C1 inhibitor treatment increased bacterial clearance, possibly through an up-regulation of CR3. Hence C1 inhibitor may be a useful agent in the treatment of pneumococcal meningitis [51]. The satisfactory outcome of the studies in animals has led various groups to assay soluble CR1 or C1 inhibitor in certain human diseases, such as the acute respiratory distress syndrome (reviewed in [52]), myocardial infarction or septic shock [53].

**BILIRUBIN: FORMATION, METABOLISM AND CYTOTOXIC EFFECTS**

UCB (unconjugated bilirubin) is a product of haem degradation by the concerted action of haem oxygenase, which converts haem into BV (biliverdin), and BV reductase, which reduces BV into UCB (reviewed in [54,55]). Under normal circumstances, UCB is conjugated in the liver with one or two glucuronic acid residues by the UDP-glucuronosyltransferase isofrom UGT1A1. The resulting glucuronides are eliminated by biliary excretion and are degraded further in the gut. When UCB production is increased (e.g. due to excessive haemolysis) and/or glucuronidation is impaired, it accumulates in blood. Upon saturation of the plasma albumin-binding capacity for UCB, the free (unbound) pigment may cross the blood–brain barrier and precipitate in specific brain areas, such as the basal ganglia (kernicterus), producing a wide array of neurological deficits collectively known as bilirubin encephalopathy. These include irreversible abnormalities in motor, sensory (auditory and ocular) and cognitive functions (reviewed in [56]).

The toxic effects of UCB in neural cells in culture include: (i) changes in cell morphology and viability, (ii) apoptotic cell death, (iii) impairment of mitochondrial function leading to uncoupling of oxidative phosphorylation, (iv) inhibition of DNA and protein synthesis, (v) changes in carbohydrate metabolism, and (vi) modulation of the synthesis, release and uptake of neurotransmitters (for a review on UCB neurotoxicity see [57]).
CYTOPROTECTIVE PROPERTIES OF BILIRUBIN RESULTING FROM ITS ANTIOXIDANT FUNCTION

A protective role for bilirubin in pneumococcal infection had already been suggested in the late 1930s [58]. Subsequently, the protective effect of bilirubin and BV against the oxidation of essential fatty acids and vitamin A was first reported by Bernhard et al. [59]. Stocker et al. [60] paved the way for the final recognition of UCB as a potent natural antioxidant with cytoprotective properties. Since then, many studies have shown that low (micromolar) ‘physiological’ serum concentrations of UCB are able to protect cells from oxidative-stress-mediated injury by scavenging ROS (reactive oxygen species), whereas at higher concentrations it generates free radicals by itself [61]. Thus UCB should be considered as a metabolically generated ‘double-edged sword’ [57] or as a ‘conditionally therapeutic’ compound [62]. Its effect, either cytotoxic or cytoprotective, will depend not only on the blood and/or tissue concentration of its free fraction, but also on the nature of the target cell/tissue, the type of insult and the cellular redox state (reviewed in [57]).

The antioxidant effects of bile pigments (BV, UCB and conjugated bilirubin) in vitro include: (i) scavenging of peroxyl radicals, (ii) inhibition of membrane lipid peroxidation (synergism with α-tocopherol by scavenging the α-tocopheroxyl radical), and (iii) scavenging of RNS (reactive nitrogen species), such as peroxynitrite or its oxidative products (for a detailed review see [61]). Interestingly, binding of bilirubin to albumin increased its capacity to scavenge peroxyl radicals, a reaction in which bilirubin is converted stoichiometrically into BV, its metabolic precursor [63].

The cytoprotective effects of bilirubin and BV have been studied in a variety of biological systems both in vitro and in vivo. Several reports have highlighted the potential value of bile pigments in preventing or treating cardiovascular and pulmonary diseases, and cancer (reviewed in [64–66]). Special attention has been given to the protection of transplanted organs, wherein ischaemia/reperfusion injury and the immune response impose serious therapeutic challenges (reviewed in [67]).

UCB, at low concentrations, protected the myocardium from ischaemia/reperfusion injury [68], reduced the generation of ROS during hypoxia in cardiac myocytes [69], prevented oxidative-stress-mediated damage in vascular smooth muscle cells [70] and endothelial cells [71], inhibited the oxidative-stress-induced chemotactic activity of monocytes [72], and suppressed vascular thrombus formation [73]. Hyperbilirubinaemia may also have a protective role in preventing hepatocellular damage in cholestasis, by counteracting bile-acid-induced apoptosis in hepatocytes and suppressing the generation of ROS by these cells [74]. BV reduced hepatocellular damage in a hepatic ischaemia/reperfusion injury model, and increased animal survival after prolonged cold ischaemia and orthotopic liver transplantation [75]. The anti-inflammatory, anti-apoptotic, antiproliferative and antioxidative properties of BV and bilirubin, and their effects on the immune response, protect transplanted organs (reviewed in [67]). BV also protected the liver from acetaminophen-induced injury by scavenging the electrophilic metabolite of acetaminophen [76], and UCB administration prevented acetaminophen-induced glutathione depletion [77]. UCB and BV also have a wide array of cytoprotective effects in the immune and neural systems (discussed in detail in [57]).

An additional facet of the protective role of UCB is manifested by its capacity to induce CYP1A1 (cytochrome P450 1A1) in jaundiced Gunn rats [78,79], thus enabling the elimination of UCB in these glucuronidation-deficient animals [80]. Low concentrations of UCB (10–50 μmol/l) induced CYP1A1 expression via AhR (aryl hydrocarbon receptor)-mediated transcriptional gene activation [81,82].

Of special importance and relevance are the studies conducted in vivo in rats with endogenously elevated bilirubin levels: the jaundiced Gunn rat, in which hyperbilirubinaemia results from a mutation in the Ugt1a1 (encoding UDP glucuronosyltransferase 1A1) gene [83–85], and the Eisai rat, which harbours a mutation in the Abcc2 gene, coding for the canalicular multi-specific organic anion transporter [MRP-2 (multi-drug resistance-associated protein 2)] [86]. By analogy, some human studies have been conducted in jaundiced subjects with Gilbert’s syndrome (reviewed in [66]). These individuals, who have a reduced prevalence of cardiovascular disease [87,88], have a congenital mutation in the promoter of hepatic UGT1A1 (UGT1A1*28 polymorphism), which results in reduced hepatic bilirubin excretion and slightly elevated serum UCB levels (26 compared with 9.7 μmol/l UCB in normal subjects). Interestingly, these jaundiced subjects have an increased antioxidant capacity which improves their resistance to serum oxidation and may explain the reduced prevalence of cardiovascular disease [89]. Thus it has been suggested that inducing moderate increases in plasma UCB concentrations may confer protection against the deleterious effects of oxidants and may contribute to the prevention and/or treatment of vascular diseases, cancer and inflammation [90].

The antioxidant radical-scavenging effects of UCB may not always be cytoprotective, as is the case in delayed subarachnoid haemorrhage-induced cerebral vasospasm, a condition associated with brain ischaemia and high mortality rates. High levels of free radicals, produced in blood clots found around blood vessels after subarachnoid haemorrhage, may oxidize bilirubin to BOXes (bilirubin oxidized products) which act on vascular smooth muscle cells causing, or contributing to, chronic vasoconstriction and vasospasm (reviewed in [91]). BOXes may be responsible for one or both events associated with cerebral
vasospasm after subarachnoid haemorrhage, including a PKC (protein kinase C)-mediated contraction and a failure to relax due to Rho-mediated inhibition of MLCP (myosin light-chain phosphatase) [92]. However, the pathophysiology of the haemorrhage-induced vasoconstriction is still not fully understood, and other aggravating factors may be involved in causing cerebral vasospasm [92], thus hindering its treatment and prevention [93].

Another example showing that the capacity of bilirubin to scavenge free radicals may not be always cytoprotective is the impairment of the bactericidal activity of neutrophils, which may lead to septic complications [57]. This example and the cytotoxicity of BOXes stress again the 'double-edged' sword character of bilirubin.

**INHIBITION OF COMPLEMENT ACTIVITY BY BILIRUBIN**

BV, at low micromolar concentrations, inhibits the complement cascade mainly at the C1 step in the CP [94], thus suggesting that bile pigments could serve as endogenous tissue protectors through anti-complement actions. BV is rapidly converted into UCB in mammals. Thus UCB may be responsible for the reported beneficial anti-complement effects of animal bile reported by Nakagami et al. [94].

We have demonstrated previously that bilirubin, mainly in its unconjugated form, inhibits the complement haemolytic cascade in sheep erythrocytes sensitized with a rabbit anti-sheep erythrocyte antibody [95]. Our study showed that the inhibitory action of UCB is exerted mainly at the C1 step of the CP, at 42.8–85.6 μmol/l UCB (UCB/albumin molar ratio, 1.42 to 2.84 respectively), and in a dose-dependent manner. The effect of UCB on C1 activity was examined by interfering either with the binding of C1 to sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody, or with the binding of C1q to a solid-phase coated with human IgM or IgG. Interestingly, the inhibitory effect of UCB was more impressive in the sensitized sheep erythrocyte model than in the solid-phase immunoglobulin model. This could result from differences in the chemical nature of the binding site of complement on different antibodies.

The haemolytic activity of the CP in vitro was also inhibited by bilirubin monoglucuronide, but to a lesser extent than that seen with UCB [95]. The UCB dianion is the major charged species interacting with albumin (Figure 4A). When bilirubin undergoes conjugation with glucuronic acid, the carboxy groups are no longer available for interaction with proteins such as C1q (Figure 4B).

To establish whether the inhibitory effects of bilirubin, as demonstrated in vitro, also applied to the in vivo situation, we evaluated further the anti-complement property of UCB in vivo through the study of its ability to prevent an acute haemolytic reaction induced by the transfusion of sheep erythrocytes into rats carrying naturally occurring hetero-antibodies capable of complement fixation [96]. UCB was infused (intravenously) into these rats and the excretion of haemoglobin in urine, which is indicative of intravascular haemolysis [18], was assessed. The UCB concentrations achieved in rat serum (15–304 μmol/l; UCB/albumin molar ratio, 0.03–0.51) produced a dose-dependent decrease in urinary haemoglobin excretion [96]. In addition, consumption of serum complement was ameliorated in these animals, confirming the inhibitory action of UCB on intravascular haemolysis.

As mentioned above, the generation of a complement-mediated immune attack may be involved in liver disorders, such as fulminant hepatitis, autoimmune hepatitis and primary biliary cirrhosis ([34,97,98], and reviewed in [99,100]), as well as in the inflammatory reactions following ischaemia/reperfusion [43]. Thus we considered it of interest to evaluate the effect of UCB on complement-mediated antibody-induced liver cell damage [101]. We found that UCB inhibited hepatocytolysis dose-dependently at concentrations ranging from 171 to 342 μmol/l (UCB/albumin molar ratio, 0.16–0.32). The anti-complement effect of UCB was tested by using an immunofluorescence assay to detect the deposition of C3 on isolated rat liver cells pre-incubated with a rabbit polyclonal antibody developed against the hepatocyte plasma membrane. We observed that the...
Table 1  Selected examples of the cytoprotective effects of UCB

<table>
<thead>
<tr>
<th>UCB administered as a solution in/added to</th>
<th>Minimal UCB concentration producing the reported effect (μmol/l)</th>
<th>Reported effect</th>
<th>Test system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs buffer (pH 7.4)</td>
<td>0.1∗</td>
<td>Protection of the myocardium against ischaemia/reperfusion injury</td>
<td>Isolated rat heart</td>
<td>[68]</td>
</tr>
<tr>
<td>Krebs/Hepes buffer (pH 7.4)</td>
<td>0.1—10∗</td>
<td>Inhibition of AngII-induced vasoconstriction</td>
<td>Rat aortic rings</td>
<td>[84]</td>
</tr>
<tr>
<td>DMEM + 20 % (v/v) FBS</td>
<td>0.5</td>
<td>Prevention of oxidative-stress-mediated damage</td>
<td>Bovine vascular smooth muscle cells</td>
<td>[70]</td>
</tr>
<tr>
<td>RPMI 1640 + 10 % (v/v) FBS</td>
<td>1</td>
<td>Inhibition of AngII-induced oxidative stress and chemotaxis</td>
<td>Human peripheral blood monocytes cell line</td>
<td>[72]</td>
</tr>
<tr>
<td>DMEM + 10 % (v/v) FBS</td>
<td>1</td>
<td>Decreased NOS2 expression by inhibiting NADPH oxidase</td>
<td>Rat macrophages</td>
<td>[83]</td>
</tr>
<tr>
<td>M199 + 15 % (v/v) FBS</td>
<td>5</td>
<td>Prevention of oxidative-stress-mediated damage</td>
<td>Porcine aortic endothelial cells</td>
<td>[71]</td>
</tr>
<tr>
<td>Aqueous solution adjusted with NaOH to pH 8.4</td>
<td>10∗</td>
<td>Suppression of microvascular thrombus formation</td>
<td>Mouse opened cremaster muscle preparation</td>
<td>[72]</td>
</tr>
<tr>
<td>Saline (pH 7.8–7.9)</td>
<td>15</td>
<td>Inhibition of complement-mediated lysis</td>
<td>Sheep erythrocytes infused into Wistar rats</td>
<td>[96]</td>
</tr>
<tr>
<td>Veronal buffer + 5.5 % (v/v) human serum</td>
<td>34.5</td>
<td>Inhibition of complement-mediated lysis</td>
<td>Sheep erythrocytes</td>
<td>[95]</td>
</tr>
<tr>
<td>William’s E buffer + 4 % (w/v) BSA</td>
<td>50</td>
<td>Inhibition of bile-acid-induced apoptosis and suppression of ROS generation</td>
<td>Rat hepatocytes</td>
<td>[74]</td>
</tr>
<tr>
<td>Veronal buffer + 7.5 % (w/v) BSA</td>
<td>171</td>
<td>Inhibition of complement-mediated lysis</td>
<td>Rat hepatocytes</td>
<td>[101]</td>
</tr>
</tbody>
</table>

The extent of C3 deposition was significantly decreased in the presence of 342 μmol/l UCB (UCB/albumin molar ratio, 0.32) [101]. This effect was consistent with decreased lactate dehydrogenase release into the medium, indicating a decrease in complement-mediated cell lysis. Further enzyme immunoassay studies showed that UCB interferes with the binding of C1q to purified anti-rat hepatocyte membrane IgG, also in a dose-dependent manner [101].

In order to establish whether the anti-complement effects of UCB reported above are associated with a direct interaction between the pigment and C1q, we synthesized radiolabelled UCB and tested its binding to equimolar concentrations of C1q, human serum albumin and IgG [101]. UCB bound to both C1q and human albumin, but not to IgG. A differential spectrum analysis of UCB in the presence of C1q confirmed further that the pigment was able to interact with this protein, in a similar way as it does with human serum albumin. Because of the nature of the differential spectrum analysis, it is possible to conclude that the observed UCB–C1q interaction is not a mere adsorption of the pigment on the protein surface as a consequence of its high hydrophobicity, but rather results from the interaction between specific structures on these molecules. Interestingly, and in contrast with the observations for albumin, the existence of an isosbestic point in the differential spectrum data indicated the formation of a unique type of complex between C1q and UCB [101].

A variety of cytoprotective effects of UCB, as studied in vitro in the presence of albumin, are summarized in Table 1.

MODEL FOR THE INTERACTION BETWEEN UCB AND C1q

The information available on the binding of UCB to serum albumin, as well as the interaction between immunoglobulins and C1q, have assisted us in building a molecular model for the interaction between UCB and C1q which results in the anti-complement effect of the pigment. This model proposes that UCB binds to C1q via an electrostatic interaction between the negative charges present on the lactam oxygen atoms of UCB and the positively charged arginine residues in the B chain of C1q (Figure 5). The following experimental findings constitute the basis for the proposed model. (i) UCB binds to basic (positively charged) amino acid residues present in human serum albumin. Lysine residues in albumin play an important role in the high-affinity binding of UCB by providing salt linkages and stabilizing the native conformation of the bound pigment [102]. Chemical modification of histidine, arginine and tyrosine residues in human serum albumin reduces the UCB-binding affinity [103]. It has been proposed that UCB...
(A) Two basic residues in the B chain of the complement component C1q (Arg\(^{114}\) and Arg\(^{129}\)) have been implicated in the interaction with negatively charged residues in the Fc portion of IgG (Asp\(^{270}\) and Glu\(^{318}\)) or IgM (Asp\(^{417}\) and Glu\(^{418}\)). This binding triggers the activation of the CP of complement system. (B) The inhibitory action of UCB on complement-mediated lysis could result from a direct interaction of the carboxy groups of the pigment with the basic residues (Arg\(^{114}\) and Arg\(^{129}\)) of the B chain of the globular head of C1q. Such binding would impair the activation of the complement system via the CP.

binding to albumin involves electrostatic interactions between positively charged amino acid residues, mostly arginine or lysine residues, and the negative charges on the lactam oxygen atoms of UCB [104,105]. In this respect, Ostrow et al. [106] have suggested that the bilirubin dianion, in which the two carboxy groups are ionized, is the charged UCB species that is preferentially bound to albumin. (ii) We have demonstrated unambiguously that UCB interferes with the normal interaction between C1q and immunoglobulins such as IgG or IgM [95,101]. As immunoglobulins bind to the globular heads of C1q [107], it is possible to speculate that UCB interacts with C1q at or near these globular heads. (iii) The structure of C1q at its globular heads is basically determined by three different modules named A, B and C [1]. These modules are tightly associated in a compact, almost spherical heterotrimeric, assembly held together by non-polar interactions, and with a Ca\(^{2+}\) ion bound at its top (Figure 6). The three modules have clear differences in their electrostatic surface potentials, which in part explain their specificity in terms of ligand recognition [108]. Modules A and C have a combination of basic and acidic residues scattered on their external face, whereas module B has a predominance of positive charges, including two arginine residues (Arg\(^{114}\) and Arg\(^{129}\)) that have been implicated in the C1q–immunoglobulin interaction [108–110]. These positively charged residues interact with two

Figure 5 Molecular model for the interaction of UCB with C1q

(A) Two basic residues in the B chain of the complement component C1q (Arg\(^{114}\) and Arg\(^{129}\)) have been implicated in the interaction with negatively charged residues in the Fc portion of IgG (Asp\(^{270}\) and Glu\(^{318}\)) or IgM (Asp\(^{417}\) and Glu\(^{418}\)). This binding triggers the activation of the CP of complement system. (B) The inhibitory action of UCB on complement-mediated lysis could result from a direct interaction of the carboxy groups of the pigment with the basic residues (Arg\(^{114}\) and Arg\(^{129}\)) of the B chain of the globular head of C1q. Such binding would impair the activation of the complement system via the CP.

Figure 6 Structure of the globular domain of C1q

Schematic ribbon representation of the globular domain of C1q seen from its top. The three modules that form the globular head of C1q are shown as strands (β-sheets) and loops arranged clockwise in the order A, B and C when viewed from the top. The Ca\(^{2+}\) ion is shown as a yellow sphere bound to the apical side of the heterotrimer.
negatively charged residues located in the Fc region of the immunoglobulin molecules, Asp
\(^{272}\) \([108]\) and Glu
\(^{318}\) \([111]\) in the Fc portion of IgG, and Asp
\(^{117}\) and Glu
\(^{118}\) in the Fc of the IgM molecule \([109]\). Kishore et al. \([112]\) expressed the globular head region of the C1q B chain as a soluble fusion protein with MBP (maltose-binding protein) in \textit{Escherichia coli}. A C1q-dependent haemolytic assay was performed to study the inhibitory effect of this fusion protein on C1q-mediated complement activation. These authors \([112]\) found that the fusion protein inhibited the C1q-dependent haemolysis of IgG/IgM-sensitized erythrocytes, this effect being more potent for IgG than for IgM. This clearly implicates module B as a putative key factor involved in the interaction of C1q with negatively charged chemical structures. Interestingly, DNA is capable of interacting with C1q and, more specifically, with the C1q globular heads, thus decreasing its binding to IgG \([113]\). As both DNA and UCB are negatively charged, it is possible that they share with immunoglobulins the capability of interacting with C1q at its globular heads and, more specifically, at the B module. This hypothesis is supported by the experimental evidence that bilirubin monoglucuronide, which exhibits a single carboxylic group available for interaction with positively charged amino acid residues on C1q, has a substantially lower ability to inhibit the activation of complement in the CP when compared with UCB \([95]\).

**INHIBITION OF COMPLEMENT ACTIVITY BY HYPERBILIRUBINAEMIA: BENEFICIAL OR HARMFUL EFFECT?**

The anti-complement properties of bilirubin may ameliorate complement-mediated damage in diseases in which antibody-dependent complement-mediated cell injury is involved, such as autoimmune haemolytic anaemias \([114]\), acute hepatitis B \([115,116]\) or hyperacute rejection of ABO-incompatible liver allografts \([117]\). Interestingly, hyperbilirubinaemia is a common alteration in these particular diseases, with levels of UCB in serum compatible with significant anti-complement action, as demonstrated by our group \([95,101]\).

The effects of UCB on the complement system are an additional demonstration of the cytoprotective properties of this pigment. However, taking into account the participation of complement in several phases of mammalian defence against bacterial and viral invasion, it is also reasonable to assume that hyperbilirubinaemia may adversely contribute to increase the susceptibility to infection. This may be the case in situations of deficiencies in complement, such as in chronic liver disorders \([118]\) or in the newborn infant \([119]\). At present, no studies have yet been carried out in patients for evaluating such possibilities.

**CONCLUSIONS**

In the present review, we provide evidence for both the beneficial and harmful effects of UCB. The effects of bilirubin on activation of the complement system are described in detail, and a molecular model for the interaction between UCB and C1q is shown.

Hyperbilirubinaemia occurring concomitantly in disorders produced by uncontrolled complement activation may represent a compensatory mechanism leading to the attenuation of cell damage. Exploring the mechanism by which bilirubin and, better still, structurally related molecules leading to complement inhibition with no potential deleterious effects may be of therapeutic interest. Indeed, it is possible that the inhibition of the complement system will turn out to be an accepted therapeutic procedure in the future. The first applications will probably be seen in those situations in which an inhibition of the cascade is needed for only a short period of time, as is the case of cardiopulmonary bypass, xenotransplantation, myocardial infarction or acute respiratory distress syndrome. The great challenge for the future is the treatment of chronic diseases that require a prolonged inhibition of the complement system, as depriving the body of this critical component of natural immunity could increase the susceptibility to infections. Thus the study of the modulation of the anti-inflammatory properties of complement provides an attractive and yet poorly explored option for the treatment of several diseases.

**ACKNOWLEDGEMENTS**

We thank Dr Guillermo Picó for his valuable suggestions, and Dr José M. Pellegrino for his help in preparation of the Figures.

**FUNDING**

The authors’ work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT number 05-26306); the Consejo Nacional de Investigaciones Científicas y Técnicas (PIP number 6442); the Universidad Nacional de Rosario, Argentina; and Yedidut Foundation in Mexico (to J.K.).

**REFERENCES**


53 Najib-Farah (1937) Defensive role of bilirubinemia in pneumococcal infection. Lancet 1, 505–506


Trepo, C., Robert, D., Motin, J., Trepo, D., Sepetjian, M. and Prince, A. (1976) Hepatitis B antigen (HBSAg) and/or antibodies (anti-HBS and anti-HBC) in fulminant hepatitis: pathogenic and prognostic significance. Gut 17, 10–13


Received 21 October 2008/27 May 2009; accepted 29 May 2009
Published on the Internet 12 October 2009, doi:10.1042/CS20080540