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Therapeutic Manipulation of the Complement System

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Abstract: The complement system is essential for host defence and tissue homeostasis. It acts largely by inflammatory reactions mediated directly by activated components and indirectly by other inflammatory systems triggered by complement. A consequence of this inflammation is destruction of micro-organisms, but frequently also damage to host tissues. Thus, complement is a double-edged sward; if activated improperly or excessively, it may cause considerable organ damage. A number of inflammatory diseases are associated with enhanced complement activation and recent data obtained from animal studies indicate that complement is in fact an important mediator in the pathogenesis of many of these conditions. An attractive approach has therefore been to develop complement inhibitors for possible therapeutic use. Such inhibitors include small molecules, naturally occurring and recombinant regulatory proteins, and monoclonal antibodies. The actual component(s) to be inhibited and the mode of application depend on the pathogenesis of the disease. Pro-drugs and targeted inhibition are to be considered for optimal benefit and minimal side effects. Despite the large body of evidence obtained from animal studies showing that complement inhibition markedly improves mortality and morbidity in a number of inflammatory conditions, it remains to be shown whether complement inhibition will be applicable in clinical medicine.

Key words: Complement inhibitors, therapy, sCR1, DAF, C1-INH, antibodies

1. INTRODUCTION

The complement system is a double-edged sword. An intact complement cascade is required for protection against infection and for maintaining the inflammatory homeostasis in the body, whereas improper, excessive or uncontrolled complement activation is disadvantageous to the host. Thus, there are two main aspects of complement pathophysiology where manipulation of the system is justified. First, genetic complement deficiencies, although rare, are frequently associated with serious diseases and substitution therapy to restore the defect function may be desired. Second, uncontrolled complement activation contributes to tissue damage in

a number of disease conditions and its inhibition could be a therapeutic approach for these diseases.

Substitution therapy with purified C1 inhibitor or plasma to treat patients with hereditary angioedema has been used for decades. Double-blind placebo-controlled studies have clearly demonstrated its clinical efficacy both in acute treatment and prevention (1). Other complement deficiencies have occasionally been treated with plasma since purified components for therapeutic use are not available. Factor H deficiency associated with haemolytic uremic syndrome (2) and C2 deficiency with systemic lupus erythemaytosus (3) have been successfully treated with plasma. Recently, a patient with factor H deficiency and hemolytic uremic syndrome with renal failure was treated by combined liver and kidney transplantation, where the defect factor H was restored by a normal protein synthesized by the liver (4). In one case of factor I deficiency, complement function was restored by administration of purified factor I (5). However, except for C1 inhibitor treatment of patients with hereditary angioedema, there are no current established regimens for substitution therapy to patients with other complement deficiencies.

Inhibition of excessive or improper complement activation has appeared to be an attractive approach to treat a number of diseases, which in animal models have been demonstrated to be totally or partly mediated by complement activation. The list of such conditions is growing and the question "In which conditions does complement activation contribute to the pathophysiology?" may be changed to "In which conditions are complement not involved?" Currently, complement activation is implicated in numerous disease conditions, e.g. ischemia-reperfusion (I/R) injury locally manifested as infarctions or systemically as a post-ischemic inflammatory syndrome, systemic inflammatory response syndrome (SIRS) and acute respiratory distress syndrome (ARDS), septic shock, trauma, burns, acid aspiration to the lungs, immune complex diseases like rheumatoid arthritis and systemic lupus erythematosus, various renal diseases, a number of inflammatory diseases in the nervous system, arteriosclerosis and transplant rejection. In principle, when inflammation is involved in the pathogenesis, complement should be considered as a possible mediator in the disease process.

This chapter is focused on therapeutic complement inhibition, with emphasis on different approaches for development of inhibitors, site of action in the cascade, possible disease conditions for complement inhibition based on experimental animal data, and finally the potential side effects of such treatment. Due to the vast amount of data already available in the literature, only parts of it can be included here. For further reading, see (6) and some selected reviews from the last 5 years on this topic (7-34).

2. GENERAL ASPECTS OF COMPLEMENT INHIBITION THERAPHY

2.1 Complement Inhibition in Human Disease

A general principle for any patient treatment is “primum non nocere” – first of all do not harm. Thus, targeting complement as a therapeutic goal requires detailed knowledge about the activation mechanisms and mediators responsible for the inflammation, enabling optimal inhibitory treatment for the actual condition. Many mechanisms have been experimentally elucidated, particularly in I/R injury, but the field is still in its infancy. Due to species differences, results from animal studies are not necessarily applicable to humans. Results from knock-out mice studies have been very useful in elucidating the pathogenic role of complement in various diseases. Successful treatment with a specific complement inhibitor is the ultimate proof that complement plays an essential role in the actual disease. The design of clinical trials has to rely on the data from the animal disease models. At the end, application of complement inhibitors to patients will reveal to what extent the various diseases will benefit from the treatment. In fact, the latter will be the ultimate concept validation for the role of complement in the pathogenesis of human diseases.

Activation of complement, as detected by increased levels of complement activation products in plasma samples, is known to occur in a number of disease conditions. However, increased complement activation does not necessarily imply that complement is of importance in the pathogenesis. On the other hand, normal systemic levels of activation products is seen in many conditions where local complement activation is likely to play a role in local tissue damage. In such conditions complement activation can be detected in tissue biopsies or body fluids. In general, an ongoing systemic activation is required for increased activation products to be detected in a plasma sample. Whether the complement activation is local or systemic will determine the inhibitor to be used and its route of application.

2.2 Complement and the Inflammatory Network

The pathophysiology of inflammation is complex and diverse, depending on the triggering factors. The role of complement in the inflammatory network may also vary with the different conditions, from being crucial to just epiphenomenonal. Inflammatory mediators mutually interact. The most important issue is whether complement activation, by activation of

leucocytes, endothelial cells and platelets, induces secondary inflammatory mediators which may contribute to the tissue damage, like cyto- and chemokines, reactive oxygen species, arachidonic acid metabolites and expression of adhesion molecules. On the other hand, some of these mediators may be primarily induced and activate complement as a secondary mechanism of inflammation. The question frequently raised is what comes first, the chicken or the egg. This has significant implication to the rationale and design of anti-inflammatory therapy in general and for complement inhibition in particular. In principle, it would be most effective to block upstream of the inflammation cascade, e.g. the primary inducer(s). The matter is, however, rather complex since some of the inflammatory mediators may have mainly adverse effects on tissue homeostasis whether others are beneficial. Thus, a major task is to identify and inhibit those mediators contributing to the tissue damage and to spare those which are beneficial. This is vital to designing a clinically optimal therapeutic regimen based on the manipulation of complement activity.

2.3 The Complement Cascade and Sites of its Therapeutic Inhibition

Fig. 1 presents a scheme of the complement cascade with an updated map of sites of inhibition (35). In brief, the complement system comprises more than 30 proteins acting together in a specific manner to protect the host against invading organisms. The *classical pathway* (upper left in Fig. 1) is activated when natural or elicited antibodies bind to antigen. C1q triggers the serine proteases C1r and C1s, the latter cleaving C4 to C4b, which exposes a specific binding site for C2. C1s then cleaves C2 and the resulting C3 convertase C4b2a cleaves C3 to C3b to form the C5 convertase C4b2a3b. Splitting of C5 to the highly potent anaphylatoxin C5a and the C6-binding fragment C5b is the last enzymatic step in the cascade.

Activation of the *lectin pathway* (Fig. 1, upper middle) is initiated by mannose binding lectin (MBL) recognising mannose on bacteria. In addition, this pathway can be activated by IgA and probably by structures exposed by damaged endothelium. MBL is homologous to C1q and triggers the MBL associated serine proteases (MASPs), of which three forms (MASP1, MASP2 and MASP3) have been described. Further lectin pathway activation is virtually identical to classical pathway activation forming the same C3 and C5 convertases. In addition there is some evidence that MASPs under some conditions may activate C3 directly.

The *alternative pathway* (Fig. 1, upper right) activation mechanisms differ from the classical and lectin pathway. Under normal physiological conditions the C3 molecule undergoes a low-grade spontaneous hydrolysis

of the internal thiol-ester and thereby binds factor B, which is cleaved by factor D and a C3 convertase is formed containing the whole C3 molecule (C3(H2O)Bb). This complex then cleaves C3 to C3a and C3b. The latter binds factor B, which is cleaved by factor D and the second alternative pathway C3 convertase C3bBb is formed. Properdin (P), the only regulator of complement which amplifies activation, binds to C3bBb and stabilises this complex, which then cleaves C3, binds C3b and the C5 convertase C3b3bBbP is formed and cleaves C5 in the same manner as the classical/lectin pathway C5 convertase.

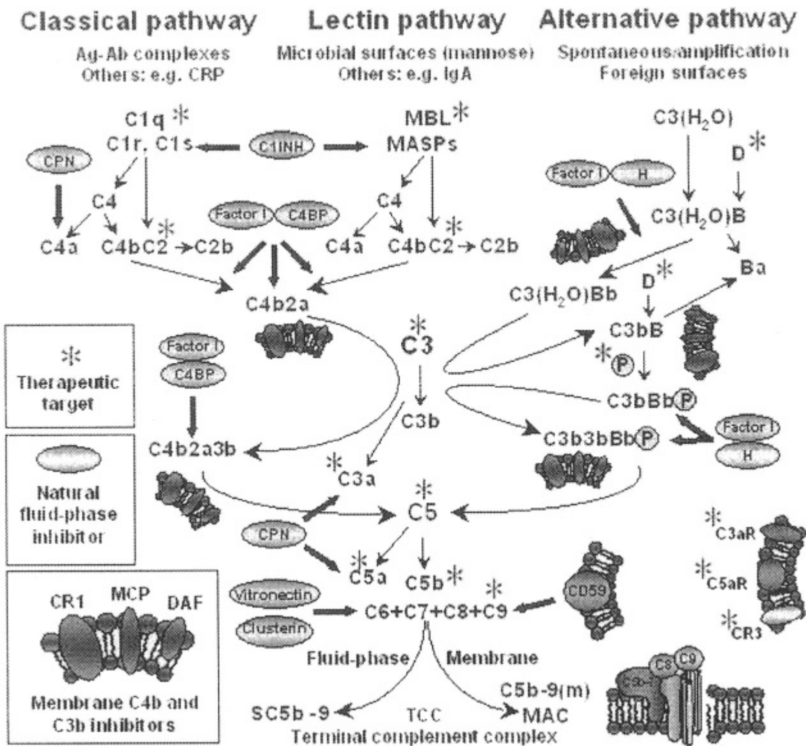

Figure 1. The complement cascade and sites of its therapeutic inhibition. Reprinted from Trends In Immunol., Vol. 23, Mollnes, T.E., Song, W-Ch., and Lambris, J.D., Complement in Inflammatory Tissue damage and Disease, pp. 61-64, Copyright (2002), with permission from Elsevier.

The *terminal pathway* (lower middle) proceeds in the same way irrespective of the initial pathway activation by assembly of C7 to C5b6,

forming an amphiphilic complex able to insert into a lipid membrane. One C5b-7 moiety binds one C8 and one or more C9 molecules, creating a physical pore penetrating the membrane (C5b-9(m) or membrane attack complex (MAC)), leading to transmembrane leakage and subsequent cell activation, or more infrequently to lysis (lower right). If the activation occurs in the fluid-phase and there is no membrane present, the C5b-7 complex binds to vitronectin and clusterin (fluid-phase regulators of the terminal pathway) and thus retains hydrophilic properties. Final assembly of a soluble C5b-9 (SC5b-9), the second form of the terminal complement complex (TCC), occurs by binding of C8 and C9.

Complement activation is strictly regulated by inhibitory proteins. In the fluid-phase C1-inhibitor (C1INH) controls C1r, C1s and MASPs whereas carboxypeptidase N (CPN) inactivates the anaphylatoxins C5a, C3a and C4a by splitting off the terminal arginine. Factor I cleaves and inactivates C4b and C3b and uses C4b-binding protein (C4BP) as co-factor in the classical/lectin pathway and factor H in the alternative pathway. The membrane regulators complement receptor 1 (CR1; CD35), membrane co-factor protein (MCP; CD46) and decay accelerating factor (DAF; CD55) regulate complement activation by either acting as co-factors for factor I mediated cleavage of C4b and C3b (CR1 and MCP), or accelerating the decay of the bimolecular C3 and C5 convertases (CR1 and DAF). CD59, also a membrane regulator, prevents the binding of C9 to the C5b-8 complex in the terminal pathway. CR1 and MCP are transmembrane proteins whereas DAF and CD59 attach to the cell membrane via a glycosylphosphatidylinositol anchor. Many of the biological effects induced by complement activation are mediated by membrane receptors such as receptors for C3a (C3aR), C5a (C5aR) and iC3b (CR3; CD11b/CD18). Activated complement is a double-edged sword with undesired effects in many conditions. Thus, various reagents with potential therapeutic applications have been developed to target complement activation and function (indicated by red asterisks in Fig. 1).

The traditional discussion of complement inhibition has focused on two alternatives: blocking at the level of C3 implying a general and broad inhibition of the system, or selective blocking of C5 activation and subsequent inhibition of C5a and C5b-9 (TCC) formation. The main argument for the first approach is that if complement activation is detrimental, it is logical to completely inhibit complement activation. The opposite view is that blocking of the terminal pathway would reduce the adverse effects whereas keeping the C3 activation open would preserve important defense mechanisms against foreign pathogens. This discussion has its background in arguing for either of the two main products developed for clinical use, namely sCR1 and anti-C5. In fact, both arguments over-

simplify the situation and have limited clinical validation. The mechanisms of complement activation and the contribution of this activation in the pathophysiology of different clinical conditions are so diverse that a differential approximation to this issue is required and several strategies must be considered.

One such strategy will be to target the initial event in the activation. This will require detailed knowledge on the activation mechanism. Each of the three initial activation pathways can be blocked separately. In the classical pathway (CP) mediated activation inhibition of C1 blocks the very first activation step and prevents the formation of C4 and C2. Similarly, blocking of mannan binding lectin (MBL) will inhibit lectin pathway (LP) activation at the step before C4 activation. A possible role of other proteins like ficolins in activation of the MBL-associated serine proteases (MASPs) must, however, be considered. Inhibition of MASP 2 would probably inhibit any LP-mediated activation. Targeting C2 will block both CP and LP, but will not prevent C4 cleavage. It has been suggested that MASP 1 may activate C3 directly, in which case blocking of C2 would not influence activation via the LP. It is, however, not likely that such a mechanism is operative *in vivo*. Inhibition of factor D, the rate-limiting component in the alternative pathway (AP) is an attractive approach in inhibiting AP activation. It should be noted that AP activation may either be primary or induced by CP or LP activation for amplification. Although inhibition of AP could block the amplification of complement activation, certain CP and LP activity important for normal functions should still be intact. In certain conditions of systemic complement activation the AP amplification loop may be responsible for an uncontrolled and detrimental activation, irrespective of the initial pathway activation mechanism. Inhibition of factor B and properdin are alternative approaches for blocking the alternative pathway. The former will require higher amount of inhibitor and the latter may not be complete, since properdin stabilizes but is not absolutely required for AP convertase activity.

A second strategy will be to inhibit the common components C3 or C5. C3 can be inhibited either by blocking activation of C3 or by inhibiting the C3 convertases. This will give a potent and broad inhibition of the whole cascade irrespective of the initial pathway. Although both C3 and C5 activation are prevented, C1/MBL, C4 and C2 will be activated. The immunomodulatory effects of C3 as well as C3 opsonization will be impaired, but may not be a concern during short-term therapy. Inhibition of C5 activation will leave the C3 functions open but block the formation of C5a and TCC. This may be beneficial in conditions where both of these terminal pathway products are involved in the pathogenesis.

A third strategy will be to target specific activation products or their respective receptors, particularly the anaphylatoxins C3a or C5a. In the case

of airway hyper-responsiveness there may be an indication for selective blocking of C3a function, which can be achieved by anti-C3a antibodies or C3aR antagonists, leaving the rest of the C3 functions open. Similarly, if C5a is the main contributor to the pathophysiology of systemic complement activation, C5a function could be blocked, leaving the C5b-9 pathway open for killing of bacteria such as Neisseria.

The main challenge for developing effective and safe anti-complement therapeutics is to balance the beneficial effects obtained by the inhibition with the preservation of sufficient functional activity for microbial protection and for tissue homeostasis.

2.4 Possible Adverse Effects

The adverse effects of inhibition of complement may be directly related to the function of complement, i.e. increased susceptibility to infection and autoimmune- and immune complex diseases, due to impaired opsonisation, antigenic responses, tolerance and handling of immune complexes. To date, such complications have not been observed in animal models. This could be attributed to incomplete inhibition and short duration of the studies. In fact, 60% inhibition of complement activity was sufficient for treatment of collagen-induced arthritis (36). This is most important when considering long-term treatment in chronic diseases. A certain degree of inhibition may be sufficient to reduce detrimental effects of complement activation, though defence mechanisms may still be preserved. The risk of infectious complications is suggested to be highest when blocking C3. The redundancy of the three initial activation pathways would reduce the risk of infection if one pathway is selectively blocked. Furthermore, blocking of C5b-9 formation could lead to increased susceptibility to Neisserial strains. Paradoxically, septic shock is one of the conditions that may benefit from complement inhibition. In these cases the patient would be appropriately treated with antibiotics and thus short-term inhibition of complement may be acceptable.

The inhibitors could be immunogenic, leading to an immune response and loss of function. The risk of antigen response is lowered by use of recombinant human proteins, small molecular inhibitors and humanized antibodies, as discussed below. Recently, a novel role for complement in tissue regeneration has been demonstrated (37). It is unknown whether impairment of this function will be a consequence of long-term complement inhibition.

2.5 Costs

Production of recombinant proteins and antibodies in eukaryotic cells is costly, whereas bacteria-produced products can be produced in large-scale at potentially lower costs. Production of small molecule inhibitors is in general economical. Costs should be considered in the context of the severity of the disease and the duration of treatment. Thus, high-cost short-time intensive treatment to save lives may be acceptable, whereas life-long treatment for chronic diseases will require low-cost regimens.

3. THERAPEUTIC STRATEGIES

Complement therapy is not only a question of which drug to be given, and at which step it interferes, but also in which form the drug is produced and administered.

3.1 Mode of Administration

The administration route is critical for chronic diseases requiring long-time therapy. Oral, nasal or rectal application is definitely superior to intravenous injection in these cases, whereas acute, severe illness is preferentially treated through the intravenous route. Furthermore, although systemic diseases like sepsis and SIRS may require systemic administration, diseases with organ specific damage, like glomerulonephrtis, central nervous system diseases and rheumatoid arthritis may benefit from local application.

3.2 Clearance and Long-Term Treatment

Recombinant proteins and small molecular inhibitors, in contrast to antibodies, generally have a short half-life and are best suited for treatment of acute conditions. Modifications to increase half-life of recombinant proteins have however been made with considerable success. Thus, conjugating the proteins to human immunoglobulin Fc fragments could increase the half-life considerably (38). This has immediate consequences for the application of the drug in long-term treatment, although the ultimate goal in these cases will be oral administration.

3.3 Targeted Application

The intention of targeted application is to deliver the drug to a specific cite for limiting the potential systemic side effects. This approach is

indicated if no systemic fluid phase activation is going on. If activation occurs systemically, inhibition should not be targeted but achieved by an agent kept soluble intravascularly.

Targeting may be nonspecific, directed to any membrane, or specific, directed to certain cells or organs. One approach for nonspecific targeting is the membrane "tagging", obtained by coupling the inhibitor to a lipid tail. This tail will bind to membranes undergoing internal-external changes, as illustrated by the myristoyl-electrostatic switch paradigm where a truncated form of sCR1 (APT070) was used (28, 39). This agent was 100-fold more potent than the parent molecule and has been used for treatment of experimental rheumatoid arthritis (40). Based on the promising experimental results, the agent has been tested in volunteers. Clinical studies in patients with rheumatoid arthritis are underway. sCD59 has also been "tagged" in a similar manner, being 100-fold more potent than sCD59 (39). The rodent complement C3 inhibitor Crry (complement receptor 1 related gene/protein) has been "tagged" using the same approach in experimental animal studies (41).

Another approach for targeted application is antigen-specific direction using antibody-conjugates. DAF was successfully targeted to the surface of Chinese hamster ovary cells by a conjugate of DAF and IgG anti-danasyl antibody (42). A similar antigen-targeted form of sCD59 has been produced (43). Recently conjugates were made between a CR2 fragment, which will recognize sites of C3 activation, and DAF and CD59 (44). These conjugates bound to C3 opsonized cells and were more than 20-fold more efficient than the untargeted molecules in inhibiting complement activation. In a mouse model of lupus nephritis CR2-DAF but not soluble DAF targeted the kidney.

A third approach for targeted inhibition is conjugating the complement inhibitor to another inflammation-modulating molecule, exemplified by the sCR1-sLe(x) (TP20) molecule (45). sLe(x) is a ligand for E- and P-selectin and thus TP20 combines inhibition of complement and leukocyte adhesion (46). TP20 was found to be superior to unconjugated sCR1 (TP10) in reducing the tissue damage in an experimental model of cerebral stroke (47), in immune-complex mediated lung injury (48), and in I/R injury in experimental allogeneic lung transplantation (49). Furthermore, TP20 reduced myocardial (50) and skeletal muscle I/R injury (51) and moderated the acid aspiration injury in mice (52).

3.4 Prodrugs

A prodrug is inactive or has low activity until it reaches a site where it is activated. Fusion of DAF or CD59 with human immunoglobulin Fc domains markedly extend the half-life (38, 53, 54). However it was found that the

biological activity was reduced. This principle was utilized to develop a novel fusion protein with virtually no biologic activity. The protein contains a site which is sensitive for enzymatic cleavage by a metalloproteinase. After enzymatic cleavage, restricted to sites of inflammation, the biologic activity of the inhibitor is restored (29, 55).

3.5 Gene Therapy

A transgene strategy for delivery of rat complement regulators using adenovirus vectors has been developed (56). Local production of the complement inhibitor Crry by astrocytes was found to attenuate experimental allergic encephaloymyelitis (57). sCR1 was delivered by retrovirally transfected cells or by naked DNA directly to the joint and prevented progression of collagen-induced arthritis (58). A similar approach was described by Quigg et al., with delivery of the inhibitor systemically (59).

Hyperacute xenotransplant rejection is caused by binding of naturally occurring antibodies and subsequent complement activation. In order to overcome this complement-mediated rejection various transgenic animals expressing human membrane complement regulators have been developed for use in xenotransplantation (see chapter 18 in this book).

4. INHIBITORS

The complement system is normally kept under strict control by fluid-phase and membrane-bound regulatory proteins (figure 1). The need for keeping this system under control is illustrated by the fact that there are as many regulators as there are ordinary components, and that deficiency of a regulatory protein is associated with substantially disturbed homeostasis. All the regulators are inhibitors of activation, except for properdin, which stabilizes the alternative C3 convertase (C3bBbP) and thus enhance activation.

4.1 C1-Inhibitor

C1-inhibitor is a naturally occurring serine protease inhibitor and the only known inhibitor of C1r and C1s. In addition to controlling the classical pathway, Cl-inhibitor is also a regulator of MASP-1 and MASP-2 of the lectin pathway (60). Recently a novel inhibitory function on the AP was documented (61). Thus, an effect of C1-inhibitor may principally be mediated through either of the initial pathways. Furthermore, C1-inhibitor is

not complement specific but has a broad spectrum of targets including factor XIIa, kallikrein and factor XIa.

C1-inhibitor is available for clinical use as substitution therapy in hereditary angioedema (HAE). Notably, the pathophysiology of HAE is closely related to the release of bradykinin and the main effect of Cl-inhibitor is to reduce bradykinin formation through inhibition of the kallikrein/kinin system. From this point of view, HAE is not a complement-mediated disease, and it should be emphasized that the effect of Cl-inhibitor, when used for treatment of other diseases than HAE, is not necessarily complement-dependent, but may well be explained by inhibition of other proteins.

C1-inhibitor has been widely used in a number of clinical conditions. The principle of supra-physiological doses to obtain more efficient regulation has been applied, although in some cases the treatment may be regarded as substitution for acquired low concentrations. The conditions treated with Cl-inhibitor include sepsis, burns, capillary leak syndrome associated with bone marrow transplantation and IL-2 therapy, myocardial infarction, trauma and transplantation. Recently it was shown in an open-labeled clinical study that Cl-inhibitor given 6 hours after thrombolytic therapy markedly reduced the size of infarction compared to matched controls (62). C1-inhibitor will not be discussed further in the present chapter, but it is referred to recent reviews on the application of C1-inhibitor in clinical medicine (63-65).

4.2 Recombinant Proteins

The family of regulators of complement activation (RCA) comprises CR1 (CD35), CR2 (CD21), MCP (CD46), DAF (CD55), factor H and C4BP. They are all powerful inhibitors of C3 and C5 convertases, except for CR2, which may play a minor role in regulation of complement. If an extensive inhibition of complement is required, the RCA proteins are candidates since they all interfere with activity of the C3 and C5 convertases.

4.2.1 C4BP and Factor H

C4b-binding protein (C4BP) and factor H are soluble regulators of the classical and alternative C3/C5 convertases, respectively. Recombinant factor H has been produced (66). Using a glycosyl-phosphatidyl inositol (GPI) anchor, a membrane form of C4BP was constructed which protected porcine endothelial cells against attack by human complement (67). A similar approach was used to bind factors H and I to a xenosurface (68), and binding of factor H to an artificial surface abrogated the surface-induced

complement activation and thereby improved biocompatibility (69). C4BP and factor H have not been used clinically. A more likely indication for recombinant factor H would be substitution therapy in factor H deficiency rather than treating enhanced complement activation. Soluble CR1 is much more potent as fluid-phase inhibitor of C3 activation than C4BP and factor H.

4.2.2 Soluble CR1

CR1 is the only member of the RCA family having cofactor activity and decay accelerating activity both in the CP and AP (C4b and C3b). A soluble form of CR1 (sCR1) was constructed by deleting the transmembrane part of the molecule (70). sCR1 inhibited both CP and AP activation in concentrations equivalent to 1% of physiological C4BP and factor H concentration. The protein was first demonstrated to reduce experimental myocardial I/R injury by approximately 50%. The cardioprotective effects have later been confirmed in several studies (71-74).

The half-life of the original sCR1 was only a few hours. A slightly extended half-life was obtained by fusing sCR1 with an albumin-binding receptor (75). An sCR1-F(ab')2 chimeric protein extended the half-life and the technique made targeting possible (76). Improving the culture condition further extended the half-life to approximately 30 hours. Parenteral administration is required.

A variant of sCR1 which specifically inhibits the alternative pathway has been constructed by deleting the C4b binding domain, sCR(desLHR-A) (77). This protein attenuated tissue damage in experimental myocardial infarction (78, 79) and reduced the endothelium-dependent relaxation in rabbit tissue, shown to be mediated by C5b-9 (80).

Human sCR1 is immunogenic in rodents, limiting its application in animal studies. Therefore, the rodent C3 convertase inhibitor Crry, which has both decay accelerating and cofactor activity and therefore resembles CR1, has been used in two different strategies in mouse models. First, a soluble Crry was constructed and coupled to an immunoglobulin tail for injection (Crry-Ig) (53). Second, Crry was over-expressed as a soluble protein in the animal under the control of a widely expressed transgene (59). Both approaches protected mice against experimentally induced acute glomerulonephritis and the Crry protein was not immunogenic. Furthermore, transgenic expression of Crry in mice developing SLE (MRL/lpr mice) prolonged survival and attenuated the renal damage (81), later confirmed by treatment with the soluble Crry-Ig protein (82). Soluble Crry was also shown to inhibit intestinal I/R injury in mice even when administered 30 minutes after start of reperfusion (54). Recently it has become evident that control of

complement activation is of crucial importance for the placenta barrier homeostasis and that complement activation may induce fetal loss (see chapter 9 in this volume). Thus, in a mouse model of anti-phospholipid antibody-induced fetal loss, soluble Crry was protective (83).

sCR1 has been used in a number of animal disease models with convincing protective effects against tissue damage. In addition to the beneficial effect on myocardial I/R injury described above, the following I/R injuries have been attenuated by sCR1: local and remote injury in rat intestine (84, 85), gut ischemia and endothelial cell function after hemorrhage and resuscitation in rats (86), liver injury (87), and local and remote (lung) injury in skeletal muscle (88, 89).

The beneficial effect of sCR1 in autoimmune diseases has been demonstrated in the passive reverse Arthus reaction, which is a dermal vascular immune-complex condition (90), in experimental arthritis in rats (91, 92), in immune-complex and complement-mediated lung injuries and thermal trauma in rats (93). Furthermore, allergic reactions (94) and pseudoallergic reaction to infusion of liposomes (95) were attenuated by sCR1. Improvement was obtained in an experimental rodent model of ARDS (96) as well as in a guinea-pig model of asthma (97). The inflammatory reaction induced by acid aspiration to the lungs in a murine model was markedly improved by sCR1 (98).

sCR1 has proved to be efficient in treatment of experimental conditions in kidneys, including glomerulonephritis (99) and in the nervous system, like myasthenia gravis (100), experimental allergic encephalomyelitis (a model for multiple sclerosis) (101), autoimmune neuritis in rats (102) and traumatic brain injury (103).

The role of complement in xenotransplantation has been indisputable and therefore the effects of sCR1 in reducing hyperacute rejection was not surprising (73, 104-106). Coupling a mini-CR1 to a GPI anchor and incorporating it into porcine cells was protective against human complement (107). The role of complement in allotransplant rejection has been less clear, but the beneficial results obtained using sCR1 in various allotransplant models has highlighted complement as an important mechanism in allotransplant rejection as well (108-111).

Extracorporeal circulation, like haemodialysis and cardiopulmonary bypass (CPB), is associated with a complement-dependent systemic inflammatory response. sCR1 reduced lung injury and pulmonary hypertension in pigs undergoing CPB (112) and inhibited complement- and leukocyte activation in a simulated extracorporeal circulation setup (113).

sCR1 (TP-10; Avant Immunotherapeutics Inc, Needham, MA) has been awarded Orphan Drug status by the FDA and has reached clinical studies. It has been tested in patients with ARDS, acute myocardial infarction, lung

transplantation and post-cardiopulmonary bypass syndrome (114). Most of the data have been published in abstract form only, but the results of the ARDS study have been published (115). Although sCR1 inhibited complement activation, there was no significant difference between the clinical outcome in the groups and the programme is discontinued.

4.2.3 Soluble DAF and MCP

A recombinant soluble form of DAF (sDAF) was constructed and found to inhibit complement both *in vitro* and *in vivo.* It attenuated the reverse passive Arthus reaction (116), but the lack of factor I cofactor activity may limit its potential for clinical application. Similarly, a recombinant soluble form of MCP (sMCP) has been made (117). sMCP attenuated the reverse passive Arthus reaction (118) and prolonged hyperacute xenograft rejection (117). A fusion protein of sMCP and a soluble form of the low affinity human IgG receptor Fc gamma RII (CD32) was more effective in protection against xenotransplant rejection than the sMCP alone (119). Comparison between sDAF, sMCP and sCR1 showed that sCR1 was more effective than each of the other two in the classical pathway. In the alternative pathway sCR1 and sMCP had almost the same effect (118). Combination of sDAF and sMCP was more effective than each protein alone. A fusion protein of sDAF and sMCP was constructed (CAB-2) which retained both decay acceleration (DAF) and co-factor activity (MCP). CAB-2 was protective in the passive reverse Arthus reaction and in Forssman shock (120), and prolonged pig-to-primate heart xenotransplant survival (121) as well as *ex vivo* porcine-to-human heart transplant graft survival (122).

4.2.4 Soluble CD59

A recombinant soluble form of CD59, sCD59, has been constructed, but is rather inefficient in the fluid phase (123). A fusion protein of sCD59 and sDAF has been constructed (124), with the aim of combining a C3/C5 convertase inhibitor activity with a C5b-9 inhibitor. It remains to be shown whether this molecule has any clinical application. A soluble form of CD59 has been fused with a soluble form of the rodent C3 convertase inhibitor, Crry, enabling inhibition of rodent complement activation both at the C3 and C9 level (125). A selective inhibition of C5b-9 may have limited indications in clinical medicine, but certain forms of glomerulonephritis, arthritis and encephalomyelitis may depend mainly on C5b-9 formation.

4.2.5 Microbial Proteins

Microbes frequently make proteins with high homology to human complement regulatory proteins and thus protect themselves against complement attack. One such protein, vaccinia virus complement control protein, has CR1-like activity and has been studied in detail (126). It has been postulated as a candidate for complement therapy. In addition to the complement-inhibitory function this protein has a binding site for heparin and direct interferes with binding of xenoantibodies to their targets.

4.3 Antibodies

Monoclonal antibodies have the advantage of high specificity, relatively long half-life and amenability for largescale production. A main limitation is the need for parenteral administration and the risk of immunization. The latter can be reduced by humanizing the antibody, or by producing human monoclonal antibodies, as recently described for an anti-C5 antibody isolated from a human phage display library (127).

4.3.1 Anti-MBL, -Factor D and -Properdin

Several recent data indicate that the lectin pathway is of importance in the pathogenesis of I/R injury (128, 129) and in experimental septicaemia where MBL-A deficiency improved survival (130). Anti-MBL antibodies blocking the lectin pathway have been developed (131, 132). A plant lectin, Ulex europaeus agglutinin II (UEA-II), has been identified as a potent inhibitor of MBL binding and thus of lectin pathway activation (133).

Factor D is the rate-limiting component in the alternative pathway and of pivotal importance for the I/R injury in mice (134), either by direct activation or through the amplification loop. An anti-human factor D monoclonal antibody (mAb) (166-32) has been characterized (135, 136) and found to inhibit complement and leukocyte activation in baboons undergoing CPB (137). An alternative approach for inhibition of the AP is blocking of properdin, although this will not lead to complete inhibition. Anti-properdin antibodies have been used *in vitro* and the data underscored the importance of AP as amplification from CP activation (138).

4.3.2 Anti-C5

Since there are no natural inhibitors acting directly on C5, there is a particular need to develop specific C5 inhibitors. Furthermore, it has been shown *in vitro* that C5 can be activated directly by leukocyte enzymes

independent of C3 activation (139), but it is unclear whether this can occur *in vivo.* Monoclonal antibodies to mouse C5, like the mAb BB5.1 (140) were important tools to demonstrate the role of the terminal complement pathway in experimental diseases like collagen-induced arthritis (36) and lupus-like nephritis (141). Notably, such treatment of collagen-induced arthritis with anti-C5 antibody not only prevented disease onset, but also ameliorated established disease. An anti-rat C5 mAb was found to be protective in experimental myocardial infarction (142). The human anti-C5 antibody N19/8 described by Würzner et al. (143) was a breakthrough in this field. It was used to demonstrate inhibition of terminal pathway activation in an artificial cardiopulmonary bypass model where leukocyte and platelet activation was also attenuated (144) and it prevented hyperacute graft rejection in a model of porcine-to-human heart transplantation (145). Later several anti-C5 antibodies have been generated. One of these, the scFv fragment h5G1.1-scFv (146) has reached clinical trials as pexelizumab (Alexion Pharmaceuticals, Cheshire, CT). In a study of patients undergoing CPB complement inhibition was obtained with subsequent reduced leukocyte activation and postoperative complications, including cognitive defects, myocardial damage and blood loss (147). Studies in rheumatoid arthritis, idiopathic membranous nephropathy, lupus nephritis and myocardial infraction are underway, but until to date the results have been described only in abstract form.

4.3.3 Anti-C5a

C5a is the biologically most potent fragment formed during complement activation and is therefore regarded as an important target for inhibition (148, 149). In animal studies anti-C5a antibodies reduced myocardial I/R injury (150), reduced neutrophil-mediated impairment of endothelium-dependent relaxation after CPB (151), reduced local and remote injury in intestinal I/R injury (152), improved survival (153, 154) and reduced IL-6 production (155) and oxygen demand (156) in sepsis, inhibited sepsis-related thymic cell apoptosis (157, 158), alleviated symptoms of ARDS in septic primates (159) and reduced lung vascular injury following thermal trauma (160). Recently anti-C5a antibodies were shown to have direct effects on the coagulation and fibrinolytic systems in a rat model of sepsis, emphasizing a possible role of C5a in activation of other plasma cascade systems (161). Anti-guinea pig C5 and anti-C5a mAbs have also been generated for examination of the terminal pathway in experimental xenotransplantation (162).

A novel approach for C5a inhibition was recently demonstrated using a mAb reacting with the C5a moiety of the C5 molecule without inhibiting C5

cleavage (163). Thus, C5a is "pre-neutralized" before it is formed and thereby increases efficacy of C5a neutralization. This antibody has been investigated in a human blood model of Neisseria, with virtually complete inhibition of the inflammatory reaction while bacterial killing was not affected (164).

4.3.4 Anti-C5-9

Anti-C8, which blocks C5b-9 (TCC) formation without interfering with C5 cleavage, was found to reduce tissue damage in rat hearts perfused with human serum (165). Furthermore, anti-C8 inhibited platelet activation during simulated CPB (166).

4.4 Small Molecule Inhibitors

4.4.1 C1 Binding Peptides

Peptides interacting with the function of C1q has been produced form phage display libraries (167, 168). Synthetic peptides interacting with IgG binding to C1 prolonged xenograft survival (169). It should be noted that C1q is not only activated by antibodies, but by several other substances which may be of pathogenic importance in human diseases, like beta-amyloid in Alzheimers disease, and C-reactive protein with possible implications for the pathogenesis of atherosclerosis. Inhibition of C1q has recently been extensively reviewed (26). A novel highly selective small molecule C1s inhibitor (C1s-INH-248, Knoll) was found to protect against experimental myocardial I/R injury (170).

4.4.2 BCX-1470

BCX-1470 is a serine protease inhibitor which was made on the basis of the 3D crystal structure of factor D. It was found to attenuate the reverse-passive Arthus reaction (171). It was found to be safe in animal toxicity studies and was tested in healthy volunteers. BCX-1470 inhibits factor D, but also C1s and several other serine proteases, which is in contrast to the specificity of the anti-factor D mAb described above. In general, serine protease inhibitors which inhibit factor D, also inhibit other serine proteases.

4.4.3 Compstatin

A 13-residue cyclic peptide was isolated using combinatorial peptide libraries to identify C3 binding peptides (172), later characterized in detail and named compstatin (173). This peptide blocks cleavage of C3 and is highly specific for binding to C3. No interaction with other cascade proteins has been demonstrated. It was found to reduce complement and granulocyte activation in an *in vitro* model of extracorporeal circulation (174) and to protect against hyperacute xenograft rejection *ex vivo* (175). Compstatin works only in primates and therefore animal studies are limited. One study showed inhibition of heparin-protamine induced complement activation in a primate *in vivo* model (176).

4.4.4 C3aR Antagonists

A nonpeptide C3aR antagonist (SB 290157) blocking human C3aR also antagonizes rodent C3aR and was found to reduce neutrophil recruitment in LPS-induced airway neutrophilia (177). The C3a/C3aR interaction may be a candidate for therapy in asthma (178, 179). Antibodies neutralizing C3a has also been shown to abolish C3aR mediated function (180). Disrupting the C3aR showed protective anti-inflammatory effects in endotoxic shock (181).

4.4.5 C5aR Antagonists

The first peptide antagonist was the linear *N* MeFKPdCHaWdR (182). Later a cyclic peptide was made, AcF[OPdChaWR] (183), which has been extensively studied in various animal models. It reacts both with primates and rodents. Beneficial effects of this antagonist have been observed in I/R injury in small intestine (184) and in kidneys (185). It attenuated chemotaxis and cytokine production (186), endotoxin-induced neutropenia (187) and the reverse passive Arthus reaction (188). It was effective when given orally to animals with immune-complex mediated dermal inflammation (189) and experimental arthritis (190).

C5aR antagonists were also developed by screening of phage-display libraries and by site directed mutagenesis of C5a, both shown to be effective in reducing complement-mediated inflammation *in vivo* (191, 192). The dimeric recombinant human C5a antagonist, CGS 32359 (191), attenuated neutrophil activation and reduced myocardial infarction size in a porcine model of surgical revascularization (193). Another C5aR antagonist significantly reduced murine renal I/R injury (194). The tertiary structure of a unique C5a receptor antagonist was determined by two-dimensional NMR spectroscopy (195) and an antisense homology box approach was used to

design C5a antagonist peptides (196). Recently, a non-peptide C5aR antagonist active in cynomolgus monkeys and gerbils, was shown to inhibit C5a mediated neutropenia after oral administration (197).

4.4.6 RNA Aptamers

Screening of an RNA pool obtained by the SELEX combinatorial chemistry technique revealed several clones binding to and inhibiting activation of human and rat C5 (198). It remains to be shown whether these molecules are possible complement inhibitors *in vivo*.

4.5 Other Inhibitors

Numerous natural and synthetic substances have been documented to inhibit complement, but these are in general not complement specific. Only a few are mentioned here.

Nafamostat mesilate (FUTHAN; FUT-175) and gabexate mesylate (FOY) are synthetic serine potease inhibitors with a broad spectrum of targets. Nafamostate is effective in several animal disease models of I/R injury, pancreatitis, xenotransplantation and cerebral infarction, but is not complement specific. Aprotinin, a complex polypeptide with effects on coagulation and inflammation, reduces postoperative bleedings, has been regarded as a candidate for general cascade inhibition. K-76 monocarboxylic acid (MX-1) is a fungal product found to inhibit several complement components, but in particular C5 (199). Although initially regarded as a promising complement inhibitor (200-202) later studies on xenotransplantation did not show efficient effect on graft survival neither by K-76 nor by FUT-175 (203, 204).

Heparin has a number of effects on complement and coagulation. Modifications to obtain complement-inhibitory heparin without effect on coagulation have been made (205). Dextran potentiates the effects of several complement inhibitory proteins including C1-inhibitor and was found to delay *ex vivo* hyperacute xenograft rejection (206). Coating of artificial devises with heparin improves biocompatibility by reducing complement activation and subsequent inflammatory reaction (207).

High-dose intravenous immunoglobulins (IVIG) are currently used to treat various inflammatory diseases. The list of biological effects of IVIG is long, including several complement modulating activities (see chapter 24 in this volume) In fact, the effect of IVIG on complement is not directly inhibitory. IVIG act as scavenger for C1q, C4b and C3b binding, thus diverting the activation products from the target to the immunoglobulin molecules. Thus, IVIG should not be used as a primary complement

inhibitor, but its effect on complement could be an additional benefit to certain inflammatory diseases.

Cobra venom factor (CVF) is frequently on the list of complement inhibitors. It should be noted, however, that CVF is not a complement inhibitor, but a potent complement activator, which depletes C3 from plasma by activating the alternative pathway amplification loop. The activation per se may influence the results obtained, therefore specific complement inhibitors developed to date should replace CVF in animal studies.

5. CONCLUDING REMARKS

Three decades ago the role of complement in human disease was largely unknown. Two decades ago it became evident that complement activation was associated with a number of pathophysiologic conditions, although the role of this activation in the pathogenesis of the diseases was largely unknown. During the last decade studies using specific complement inhibitors or complement knock-out animals have revealed that complement plays a crucial role in the pathogenesis of tissue inflammation in a number of animal disease models. These include local and remote damage after ischemia and reperfusion, immune-complex and autoimmune diseases in general and joint, kidney and central nervous system diseases in particular, ARDS and systemic inflammatory response due to sepsis or extracorporeal circulation, antibody-mediated fetal loss, and allo- and xenotransplantat graft rejections. Based on these encouraging data a great enthusiasm evolved for treatment of human diseases using complement inhibitors. Unfortunately the progress in clinical application has been relatively slow. This may be attributed to several reasons: Human diseases may have a more complex pathophysiology than the animal models. Experimental conditions differ significantly from the clinical with respect to time for and mode of application. The number of drugs for clinical use has been markedly limited compared to those used in animal models. Targeted inhibition has hardly reached clinical application. Finally, the selection of patients and size of test groups may have been inadequate for demonstrating clinical benefit. The approach of complement inhibition for treatment of human diseases is still in its infancy, in fact it has hardly reached delivery. There is a need for more experimental studies, which should focus on the mechanisms of complement-mediated damage. This will provide a rational approach to design and optimize the treatment, i.e. identifying the component(s) needed to be inhibited in the actual condition and targeting the inhibition to the actual site of inflammation. With the advent of numerous novel complement

inhibitors, it is hopeful that the potential benefits of complement inhibition in human inflammatory diseases could be validated and realized.

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