Targeting Complement in Treatment of Intestinal Ischemia/Reperfusion-Induced Injury

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ABSTRACT

Complement activation occurs during tissue injury and inappropriate or excessive activation contributes to the expression of pathology becoming a double-edged sword. Understanding the role of complement and its natural regulatory molecules will enable the development of therapeutic interventions to prevent excessive damage during mesenteric ischemia/reperfusion (IR). In this chapter, we will briefly review the mechanism of complement activation during intestinal ischemia/reperfusion and discuss results and significance of mesenteric ischemia/reperfusion induced injury in animal models with altered complement activation. Finally, we will discuss development of current inhibitors of complement activation and those that may be used in the future.

1.0 ISCHEMIA/REPERFUSION INTESTINAL DAMAGE

Despite advances in medical care, the mortality rate for acute mesenteric ischemia remains unchanged from the 1980's at 81%. The lack of decreased fatalities is due to the fact that it is a rare and difficult diagnosis with rapid progression from local intestinal injury to systemic release of inflammatory mediators leading to distant organ injury. Intestinal ischemia is associated with multiple trauma conditions, such as hemorrhagic shock, burns, myocardial infarction and multiple organ failure [Trunage 1994]. These conditions lead to a reduction of blood volume that is believed to result in splanchnic vasoconstriction and functional if not true ischemia of the gut [Williams 1983; Austen 1999; Dong 1999; Eror 1999; Kilgore 1999; Rehrig 2001].The splanchnic circulation is a large vascular bed that receives as much as 25-30% of the total blood flow bringing oxygen and nutrients to the intestine. As blood flow to the intestine decreases, the flow to the various circuits is not decreased equally with more flow shunted to the mucosa than to other networks. The rapid turnover of the mucosa makes it extremely sensitive to hypoxia. Therefore, loss of blood flow to a tissue for a limited amount of time, as little as 20 min, results in damage to the mucosal surfaces with villi disruption. However, reperfusion induces pathological changes to the tissue that are greatly enhanced compared to that of ischemia alone. These alterations of reperfusion injury after mesenteric ischemia cause additional local inflammation characterized by complement activation and deposition, neutrophil infiltration and eicosanoid generation that coincides with mucosal injury [Eror 1999; Rehrig 2001; Conner 1999]. The role of complement in mediating this injury and the possibilities to inhibit complement activation is the focus of current research.

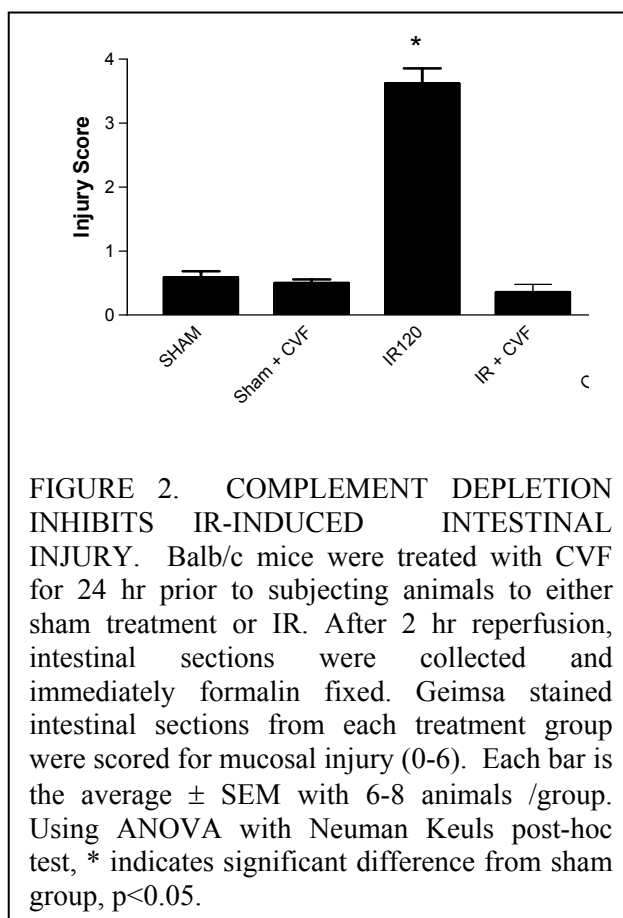## 2.0   COMPLEMENT ACTIVATION IN INTESTINAL DAMAGE

Complement is a complex cascade of over 30 proteins that are activated in an orderly manner. The cascade has 3 initiating arms: that is the classical, lectin and alternative pathways that each produces enzymatic complexes, C3 and C5 convertases (Fig. 1). The cascade continues with the cleavage of C3 and C5 and all 3 pathways culminate in a common terminal pathway. The terminating complex, the membrane attack complex, is a lytic complex that inserts into the membrane forming a pore in the cell. Since the complement pathway is capable of extreme cell and tissue damage, complement regulatory molecules that control the rate of its activation are essential and occur naturally at multiple points within the cascade. However, in many clinical conditions, unregulated complement activation and subsequent tissue damage occurs during intestinal ischemia, blunt trauma and hemorrhagic shock.

FIGURE 1. SIMPLIFIED COMPLEMENT ACTIVATION CASCADE.
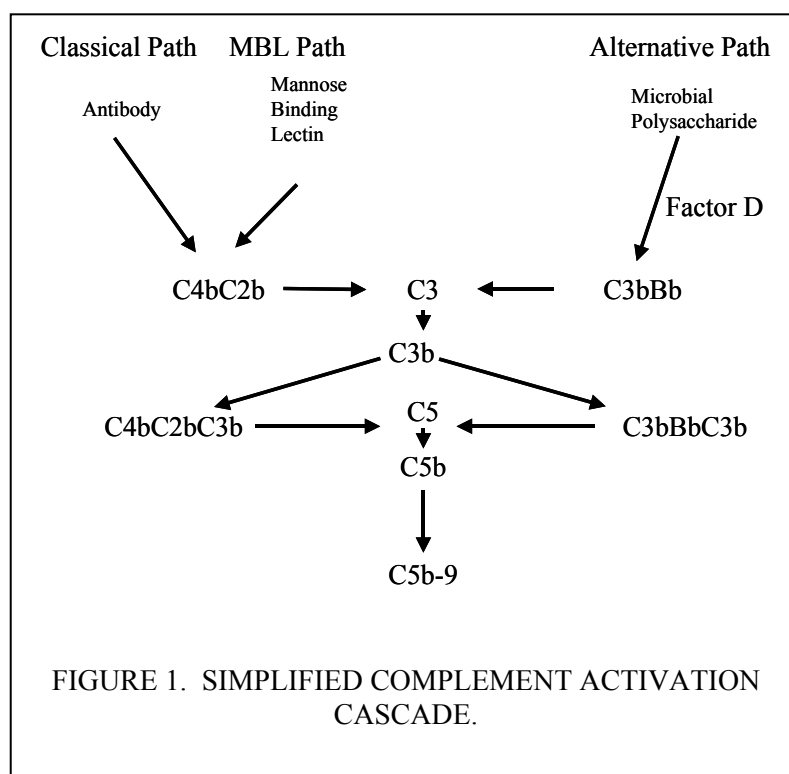
Many of the clinical conditions associated with inappropriate complement activation also reduce blood volume and lead to subsequent mesenteric vasoconstriction resulting in functional intestinal ischemia [Kilgore 1999]. When organs such as the intestine are subjected to severe vascular ischemia, followed by reperfusion of blood into the site, local as well as remote tissue inflammation and injury ensues. Intestinal damage as a result of ischemia and subsequent reperfusion varies by the region of the intestine. However, the extremes of the IR-induced injury apply to the entire intestine. In other words, the intestine can tolerate short periods of ischemia without severe injury but long periods of mesenteric ischemia followed by reperfusion results in death. Mesenteric ischemia triggers an inflammatory reaction, characterized by neutrophil infiltration,

activation and local mucosal injury, in which complement activation plays a pivotal role. Reperfusion of the ischemic gut is believed to lead to another surge of complement activation and further mucosal injury [D'Ambrosio 2001].

Using rodent models, the mechanism of IR-induced intestinal damage has been shown to involve complement activation. The exact mechanism of complement activation during intestinal IR remains unclear as there is evidence that more than one complement pathway (classical, alternative or lectin) may be activated and enhancing tissue injury [Fleming 2000; Stahl 2003; Williams 1999]. Further evidence that complement activation is directly involved in the effector phases of intestinal IR injury has been provided by studies showing that inhibition of the complement pathway at the point of C3 or C5 activation can either prevent or substantially attenuate intestinal injury [Austen 1999; Eror 1999; Rehrig 2001; Williams 1999; Hill 1992; Fleimg 2003a]. In addition, inflammatory mediators generated during complement pathway activation, such as the anaphylatoxin C5a and the membrane attack complex (MAC), are known to be able to directly cause cellular activation and injury too [Fleming 2003a; Ward 2000; Kohl 2001]. The specifics of each complement initiating pathway, the terminal complex and the anaphylotoxins will be discussed below.

FIGURE 2. COMPLEMENT DEPLETION INHIBITS IR-INDUCED INTESTINAL INJURY. Balb/c mice were treated with CVF for 24 hr prior to subjecting animals to either sham treatment or IR. After 2 hr reperfusion, intestinal sections were collected and immediately formalin fixed. Geimsa stained intestinal sections from each treatment group were scored for mucosal injury (0-6). Each bar is the average $\pm$ SEM with 6-8 animals /group. Using ANOVA with Neuman Keuls post-hoc test, * indicates significant difference from sham group, $p<0.05$.

## 3.0   INDICATIONS OF COMPLEMENT INVOLVEMENT

The essential role of complement in mesenteric ischemia/reperfusion induced tissue injury has been shown in numerous animal models. We have established a mesenteric IR model in mice and confirmed the role of complement in intestinal IR using cobra venom factor (CVF) to deplete complement factor 3 (C3). The intestinal mucosa of the sham-operated animals remained normal as indicated by an injury score of 0.68 (Fig. 2).   Mice subjected to IR had significant intestinal injury as indicated macroscopically by swollen and edematous with areas of red streaks. Microscopically, the injury ranged from shortened and vacuolated villi to complete destruction of normal mucosal architecture with frank hemorrhage. In contrast, mice subjected to sham procedure or IR after treatment with CVF had no significant intestinal mucosal injury (Fig. 1).   Thus, similar to the rat model, complement has a role in the local injury induced by IR in mice.

Activation of complement 5 (C5) leads to the production of a potent anaphylotoxin, C5a, along with C5b, the initiator of the membrane attack complex. The use of C5 deficient mice showed that either the membrane attack complex, the anaphylatoxin, C5a or a combination of both could prevent or substantially attenuate intestinal injury [Austen 1999; Wada 2001]. In additional studies, anti-C5 monoclonal antibodies have been administered to mice to prevent C5 activation and subsequent local and remote tissue damage [Fleming 2003a and 2003b; Wada 2001]. Inhibition of C5 activation by an anti-C5 antibody administered to wild type mice subjected to mesenteric IR, prevents C5a generation, PMN infiltration and deposition of the terminal complement complex on the damaged issues in a manner similar to that observed in C5 deficient mice [Wada 2001; Wang 1995; Vakeva 1998a and 1998b; Rinder 1995; Fleming 2002]. These studies however, do not distinguish between the actions of C5a and C5b-9 terminal complex or address the initiating pathways.

4.0   EVIDENCE FOR THE INITIATING PATHWAY

It is known that complement is activated immediately after injury and the severity of the trauma is directly proportional to the level of complement activation [Fodde 1998]. The complement cascade can be activated by contact with microbes but during short periods of intestinal IR, the alternative and classical pathways are both over-activated in the absence of microbial infection. It is well known that the clotting cascade activates complement.   Some possible alternative complement activators include: reactive oxygen or nitrogen metabolites, exposed collagen, mitochondrial membranes and extracellular ATP [Goris 2000; Gallinaro 1992; Mollnes 1999]. In addition, in vitro data shows that damage to the endothelium activates the alternative pathway and recent data show that the absence of Factors D or B protects mice from IR-induced damage [Stahl 2003; Fruchterman 1998].   It is possible that multiple pathways are involved in the complex mechanisms of mesenteric ischemia reperfusion induced damage. Although the exact method of complement activation may differ with the traumatic insult, the down-stream events of excessive complement activation results in an inflammatory reaction.

The ability to design logical therapeutics to prevent complement action depends on our understanding of the complement pathways that are involved and the initiating factors for the specific pathway(s). The availability of a mouse model and mice engineered to be genetically deficient in a specific complement factor has aided our understanding of the role of complement components in mesenteric injury. These studies are summarized in Fig. 3 and detailed below.

FIGURE 3. MESENTERIC IR STUDIES USING COMPLEMENT DEFICIENT MICE.
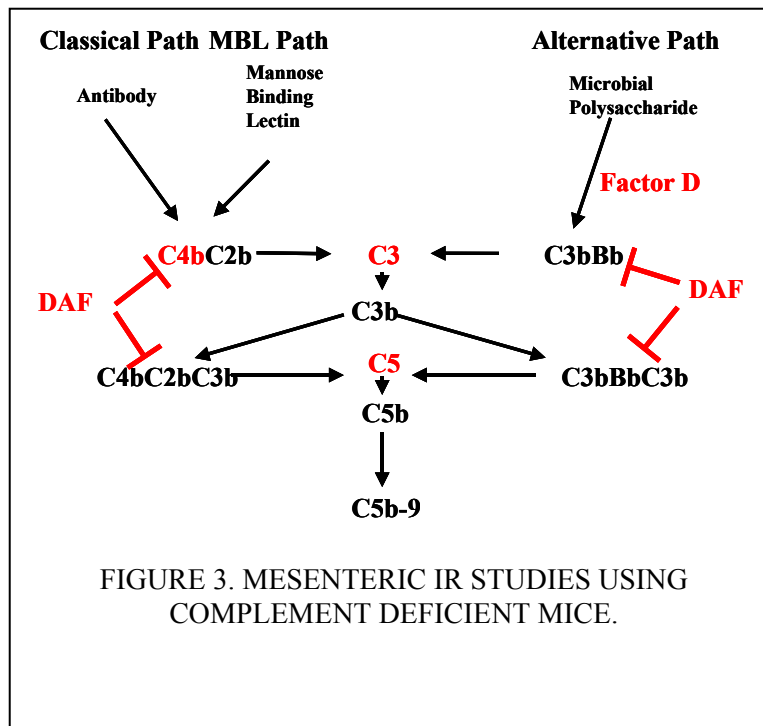
4.1 Role of Classical Complement Pathway

Three observations have strongly implicated the classical pathway in the process. The first is that intestinal IR injury is significantly decreased in *RAG-1-/-* mice, and reconstitution of these Ig deficient mice with purified IgM natural antibody to normal levels [Williams 1999] restores IR-induced injury. The second is that mice with normal levels of natural antibody, but in which the gene encoding complement C4 is inactivated (*C4-/-*), are protected from injury [Williams 1999]. The importance of natural IgM antibody and the classical complement pathway in mediating IR injury of skeletal muscle has also been shown using a similar experimental strategy with C3, C4 and Ig deficient mice [Weiser 1997]. From these and other findings, it has been proposed that natural antibodies bind to antigen(s) revealed on the surface membrane of cells subjected to ischemia and subsequently activate complement by recruiting C1 and then cleaving C4 [Williams 1999]. This is followed by the generation of complement C3 and C5 activation fragments with ensuing increases in adhesion molecule expression and release of a cascade of inflammatory mediators, including leukotriene B4 and others [Eror 1999; Rehrig 2001; Fleming 2000]. Finally, mice deficient in complement receptors 1 and 2 (CR2-/-) are also resistant to local IR induced damage In addition, injection of IgM and IgG from wild type mice restored all measured parameters of IR-induced injury indicating that role of antibody and the classical complement pathway are important in initiating complement activation.

4.2 Role of Lectin Pathway

The studies discussed above using C3 and C4 deficient mice, suggest that either the classical or the lectin pathways have a role in local IR-induced injury. There is a lack of intestinal ischemia studies using lectin

pathway deficient mice or lectin specific inhibitors. However, Stahl's group has shown deposition of MBL on hypoxic endothelial cells in vitro [Collard 1999]. In additional studies, anti-mannose binding lectin antibodies were used to determine the role of MBL in myocardial IR-induced injury [Wetsel 2000]. These studies indicate that the lectin pathway is activated during the oxidative stress associated with ischemia. Further studies will be needed to show the pathway's role in intestinal IR-induced injury.

4.3 Role of Alternative Pathway

There is recent data indicating that the alternative complement pathway is also involved in intestinal IR injury. Factor D, of the alternative pathway, forms the alternative pathway C3 convertase by cleaving Factor B. Factor D deficient mice, lacking the alternative C3 convertase, are resistant to IR-induced intestinal and pulmonary damage. Mesenteric IR-induced complement deposition is prevented in both the intestine and the lungs. Additionally, administration of Factor D restored IR-induced decrease in intestinal lactate dehydrogenase. This restoration was prevented by treatment with anti-Factor D antibodies. Thus, it appears that the alternative complement pathway is actively involved in the complex mechanism of mesenteric IR injury.

5.0 ROLE OF THE LYTIC COMPLEX OR ANAPHYLOTOXINS

C5a, a small, glycosylated peptide, is a potent chemoattractant for PMN, monocytes and T cells (reviewed in [kohl 2001; Jordan 2001]. In vitro studies have shown that C5a induces degranulation, respiratory burst, increases adhesion molecule expression and delays apoptosis in PMN [Perianayagam 2002; Tyagi 2000; Binder 1999]. In addition, *in vivo* studies using anti-C5a antibodies have indicated that C5a alters vascular permeability and neutrophil activation during cardiopulmonary bypass [Tofukuji 2000], hind limb IR [Bless 1999], sepsis [Huber-Lang 2001a and 2001b; Reidemann 2002 and 2003] and inflammatory lung injury [Mulligan 1996]. C5a receptors (C5aR) are expressed on the surface of intestinal cells under inflammatory conditions, as well as on bronchial epithelial cells [Rothermel 2000]. Blockade of these receptors using C5aR antagonists (C5aRa) indicates a role for C5a in systemic activation of neutrophils in multiple animal models [Pellas 1998; Heller 1999; Haynes 2000; Arymugam 2002]. To distinguish the role of C5a from that of C5b-9 on local and remote tissue injury, we inhibited the actions of C5a during mesenteric IR by treating wild type mice with a cyclic hexapeptide C5a receptor antagonist (C5aRa) and we administered C5a to C5 deficient (C5$^{-/-}$) mice subjected to mesenteric IR. These experiments showed that during IR, C5a is sufficient to induce limited local damage and eicosanoid production but not systemic PMN activation. In addition, systemic C5a administered during IR can induce VCAM-1 expression on remote organs such as the lung without inducing increased vascular permeability.

This inflammation involves anaphylatoxin recruitment and subsequent activation of granulocytes as well as upregulation of endothelial adhesion molecules, the local release of other inflammatory mediators and cytokines. Together these potent mediators may result in local damage or may activate the inflammatory response (and complement) systemically. Extensive systemic complement activation can lead to a whole body inflammatory reaction such as adult respiratory distress syndrome, systemic inflammatory response syndrome, and multiple organ failure.

6.0 COMPLEMENT INHIBITION OF INTESTINAL DAMAGE

Because this cascade of proteins results in cell lysis and tissue destruction, the complement system includes multiple regulatory proteins that prevent non-specific complement activation. Some of these regulatory proteins are cellular receptors for the breakdown products of the components of the system. The understanding of natural regulatory proteins and receptors and their involvement in protection of mesenteric damage has allowed the design and development of therapeutic interventions that inhibit complement activation and prevent inflammatory tissue damage. Complement inhibitors are currently being studied to determine their ability to inhibit tissue damage as a result of mesenteric IR (summarized in Fig.4). Using a rat model of intestinal IR, several groups showed that administration of sCR1, a regulator of both classical and alternative pathways, significantly reduced rat local and systemic injury, PMN infiltration, and leukotriene B_4 (LTB_4) production [Eror 1999; Hill 1992].

FIGURE 4. MESENTERIC IR STUDIES USING COMPLEMENT INHIBITORS.
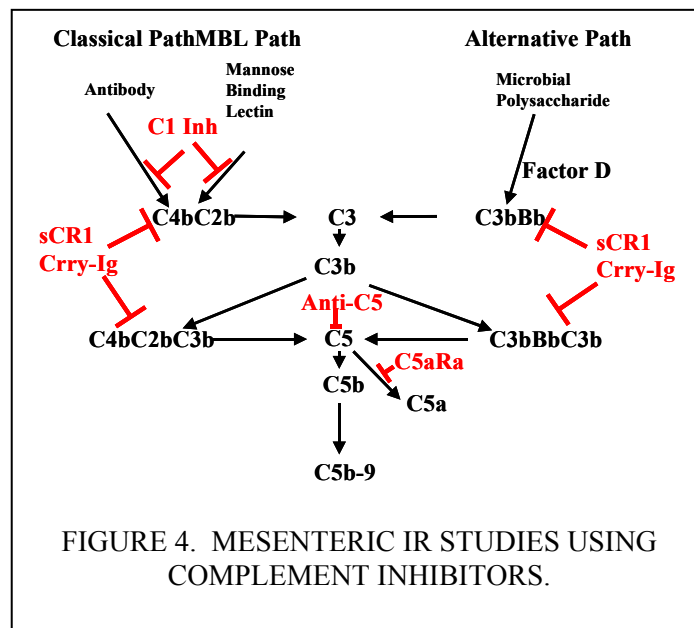
In mice, complement receptor 1-related gene/protein y (Crry) is a membrane regulatory protein altering the activity of both the classical and alternative complement pathways. Using a recombinant soluble form of Crry fused to the hinge, CH2, and CH3 domains of mouse IgG_1 (Crry-Ig), mice were pretreated either 5 min prior to, or 30 min after, the initiation of the reperfusion phase of mesenteric IR. IR-induced injury was reduced after Crry-Ig was administered. Pre-treatment with Crry-Ig reduced the local intestinal mucosal injury and decreased generation of LTB_4. When given 30 min after the beginning of the reperfusion phase, Crry-Ig resulted in a decrease in IR-induced intestinal mucosal injury comparable to when it was given 5 min prior to initiation of the reperfusion phase. Despite the presence of substantial number of neutrophils, Crry-Ig administered 30 min after the initiation of the reperfusion prevented the IR-induced tissue injury damage. This indicates that although neutrophils may have a role in the damage, complement inhibition is beneficial.

C1 inhibitor (C1 Inh) inhibits the earliest steps of the classical and the mannose binding lectin pathways. When C1 Inh was administered to mice prior to mesenteric IR, mucosal injury and was effectively inhibited in a dose dependent manner. These findings emphasize the importance of complement activation in ischemia/reperfusion and highlight the potential therapeutic use of C1 Inh in limiting and/or preventing damage caused by ischemia/reperfusion.

Because the local damage itself is not believed to be life threatening, other groups have focused on C5a as a cause of the excessive systemic inflammatory response. Using a small peptide C5a receptor antagonist that binds the human C5a receptor, it has been shown that serum markers of systemic inflammation, neutrophil activation and remote organ injury can be prevented even when the peptide is given during the ischemic period, prior to beginning reperfusion [Fleming 2003a and 2003b; Arumugam 2002].

Recently, IVIg (high-doses of immunoglobulins modified for intravenous use [Basta 1996] have successfully blocked complement mediated tissue injury in a rat model of mesenteric ischemia/reperfusion [Anderson 2001a, 2001b, and 2002]. Therefore, although the exact mechanism of complement activation has not been elucidated, it is apparent that complement plays a substantial role in both local and systemic tissue injury during ischemia of multiple organs.

7. 0   DEVELOPMENT OF THERAPEUTICS TO PREVENT COMPLEMENT-MEDIATED INTESTINAL INJURY

As indicated above, complement activation is part of the pathogenic process in mesenteric IR. The complement activation process appears to involve each of the three initiation channels as well as the common terminal pathway and anaphylotoxin in the damage process. As discussed above, animal models of mesenteric IR have clearly shown, that inhibition of complement activation can, prevent, improve or reverse the disease pathology. Naturally occurring inhibitors control the amount of injury induced by the cascade of complement activation and may become useful therapeutics. However prior to the therapeutic use of these inhibitors, a number of important questions must be answered. First, is complement central in pathology of mesenteric IR? Second, which pathway is the primary initiator of the activation? Third, is general complement inhibition associated with side effects such as suppression of the innate immunity and the appearance of overwhelming infections?

Complement inhibitors for therapeutic use in mesenteric IR are being designed in a logical fashion. First, using molecular engineering, monoclonal antibodies that block activation of central complement factors can be humanized and used in the treatment of disease. An anti-C5 antibody is in human trials for use in other diseases. Second, the half-life of natural complement activation inhibitors such as DAF, CD59, CR1 can be extended by genetically fusing with the Fc portion of IgG. Third, fusing of multiple complement inhibitors that act at different stages of the activation cascade may act at different phases resulting in more effective complement inhibition. Fourth, recently, peptide inhibitors blocking the action of convertases have emerged as a new promising approach. Compstatin represents such an example as it inhibits complement activation by blocking C3 convertase-mediated cleavage of C3 [Sahu 2003]. Fifth, and lastly, in response to the consideration that the use of complement inhibitors may cause systematic inhibition and unwanted side effects, from the complete lack of complement, such as overwhelming infection, investigators have considered the fusion of complement inhibitors to molecules that will direct it to the site of inflammation. For example, complement inhibitors can be conjugated to selectin ligands that will direct them to sites of increased selectin

expression, i.e., inflammation or delivered via targeted liposomes to a specific location where the inhibitor is released in a concentrated region.

8.0 ACKNOWLEDGMENTS

9.0 REFERENCES

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