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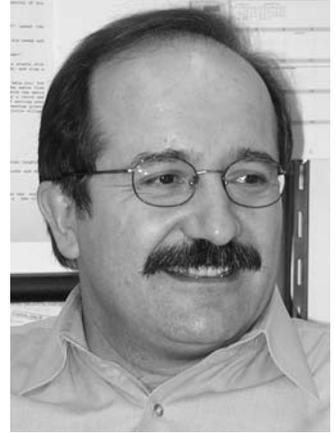
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Complement

Structure, Functions, Evolution, and Viral Molecular Mimicry



Dimitrios Mastellos¹
Dimitrios Morikis²
Stuart N. Isaacs³
M. Claire Holland¹
Cristoph W. Strey¹
John D. Lambris^{*,1}

¹Protein Chemistry Laboratory,
Department of Pathology and
Laboratory Medicine,
University of Pennsylvania,
Philadelphia, PA

²Department of Chemical and
Environmental Engineering,
University of California,
Riverside, CA

³Division of Infectious
Diseases, Department
of Medicine, University
of Pennsylvania,
Philadelphia, PA

Abstract

The complement (C') system has long been recognized as an important mediator of innate immune defense and inflammation. In recent years there is increasing evidence suggesting that complement components may also participate in non-inflammatory and developmental processes. Here we review our current work on the structural–functional aspects of C3-ligand interactions and the rational design of small-sized complement inhibitors. We present a novel, proteomics-based, approach to studying protein–protein interactions within the C' system and discuss our progress in the study of viral immune evasion strategies. Furthermore we discuss the involvement of complement proteins in organ regeneration and hematopoietic development.

Key Words

Complement; inhibitors; proteomics; immune evasion; evolution; regeneration.

Introduction

The complement (C') system consists of a complex group of serum proteins and glycoproteins and soluble or membrane-bound

receptors, which together play an important role in host defense against infection. Complement, a phylogenetically conserved arm of innate immunity (1), functions together with the adaptive immune response by serving as

*John D. Lambris
Department of Pathology and Laboratory
Medicine, University of Pennsylvania
401 Stellar-Chance Laboratories
Philadelphia, PA 19104
E-mail: lambris@mail.med.upenn.edu
Website: www.complement.us

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an important inflammatory mediator of antigen–antibody interactions (2,3). It also provides an interface between the innate and adaptive immune response by contributing to the enhancement of the humoral response mounted against specific antigens (4).

Complement can be activated through the classical, alternative, or lectin pathways. Antigen–antibody complexes initiate the activation of the classical pathway, whereas the alternative and lectin pathways are activated in an antibody-independent fashion through the interaction of complement components with specific carbohydrate groups and lipopolysaccharides present on the surface of foreign pathogens (e.g., bacteria) (5,6). Complement activation proceeds in a sequential fashion through the proteolytic cleavage of a series of proteins and leads to the generation of active products that mediate various biological activities through their interaction with specific cellular receptors and other serum proteins (6). During the course of this cascade, a number of biological processes are initiated by the various C' components, including inflammation, leukocyte migration, and phagocytosis of C'-opsonized particles and cells. One result of these C'-mediated events is a direct lysis of target cells and microorganisms as a consequence of membrane-penetrating lesions (pores). Currently, over 30 C' proteins have been identified, and deficiencies in any particular component have been frequently associated with a diminished ability to clear circulating immune complexes or fight infection (3,7). This association of C' deficiencies with disease states emphasizes the important physiologic role of the C' system in the defense against infections.

Among the C' proteins, the third complement component C3 is likely the most versatile and multifunctional molecule known, interacting with at least 20 different proteins

(5). The most abundant C' protein in serum (1–2 mg/mL), C3 is a glycoprotein that is comprised of a 115-kDa α -chain linked to a 75-kDa β -chain by a single disulfide bond and noncovalent forces. Molecular modeling of C3 based on data derived from X-ray scattering studies depicts it as a two-domain structure, with a flat ellipsoid associated with a smaller flat domain (8).

C3 is a pivotal component of the complement system, because it can be activated through all three pathways (classical, lectin, and alternative). Cleavage of C3 by highly specific enzymes termed C3 convertases leads to the generation of C3a and C3b. C3a is a peptide with anaphylatoxic properties that exerts various inflammatory functions through binding to its receptor C3aR, which is expressed in several cell types (9). C3b transiently acquires the ability to be fixed covalently to various activating surfaces, and its generation from native C3 exposes multiple binding sites for other complement components, including C5, properdin, factors H, B, and I, C4-binding protein (C4bp), CR1 (C3b receptor), and the membrane cofactor protein (MCP) (10–12). Binding of C3b to these proteins modulates complement activation by leading either to the amplification of the alternative pathway C3 convertase or to the inactivation of C3b. Further degradation of C3b generates active fragments that interact with various complement receptors and mediate important immunoregulatory and (as recently suggested) noninflammatory functions (12a). In addition, C3b participates in the assembly of the C5 convertase (13), an enzyme that cleaves native C5 to generate C5a and C5b.

C5a is a potent anaphylatoxin with chemotactic as well as cell-activating properties that are mediated through stimulation of its receptor C5aR; C5b participates in the assembly of the C5b-9 complex (MAC) that induces the

lysis of complement-targeted cells (14). Recently, it has also been shown that MAC can trigger cellular activation events and has a growth effect on cells that have been exposed to sublytic doses of C5b-9 complexes (15).

Here we review our current work on the structural–functional aspects of C3-ligand interactions and the rational design of small-size complement inhibitors. We also present further progress on our studies of viral molecular mimicry and immune evasion strategies, as well as our recent work on evolutionary aspects of complement biology. We discuss novel associations of various complement components with developmental pathways and present our research on the role of complement in tissue regeneration and early hematopoietic development.

Studies on Protein–Protein Interactions of Complement Components

Computational Studies

Computer simulation is a quantitative tool to understand the underlying chemistry and physics of important biological processes both microscopically and macroscopically. The availability of three-dimensional protein structures from crystallographic or nuclear magnetic resonance (NMR) studies provides us with atomic level detail of the individual elements of the protein, as well as with the properties of the surfaces or volumes of the protein as a whole. An increasing number of structures of protein complexes deposited at the protein data bank and comparison with the structures of free proteins allow for insight into the structural changes occurring on association and into the forces that drive recognition and binding. The types of interactions responsible for the formation of the interfaces of protein complexes are hydrophobic interactions among nonpolar side chains, hydrogen-

bonding interactions, electrostatic interactions of ion pairs (salt bridges), and steric interactions. Additionally, electrostatic complementarity of the molecular volumes and shape complementarity of the molecular surfaces provide the means for recognition and selectivity from alternative association configurations. Finally, the presence or exclusion of solvent molecules in molecular interfaces contributes to the stability of the association.

We have initiated theoretical studies with the aim of understanding the biophysicochemical nature of the interactions that involve complement components. The nature of the interaction between complement component C3d and complement receptor type-2 (CR2) has been a subject of intense study and speculation. This interaction depends strongly on the solution ionic strength, an observation that is indicative of the involvement of electrostatics in the association. The recent solution of the three-dimensional structure of the complex of C3d–CR2 (SCR1/SCR2) has revealed the sites of the association of the two molecules, which are dominated by the presence of hydrophobic and steric interactions, as well as hydrogen bonds (16). To understand the role of charges in C3d–CR2 association, we have performed electrostatic calculations based on the solution of the finite-difference linearized Poisson–Boltzmann equation with continuous solvent. We have used the molecular detail of crystallographic structures of free C3d (17) and C3d–CR2 complex (16) to perform our calculations at the atomic level. The electrostatic interactions among all ionizable residues of C3d and C3d–CR2 have been determined in the form of electrostatic potentials, which have been converted into ionization free energies and, ultimately, into apparent pK_a values. The latter provide us with a quantity that can be easily interpreted both theoretically and experimentally (Morikis and Lambris, in

preparation). Our studies suggest that the association of C3d with CR2 is electrostatic in nature and involves the whole molecules and not only the limited association sites. These results are in agreement with mutagenesis studies on C3d within the association site (16) and explain the effect on binding of additional mutagenesis studies away from the association site (18). Our results also account for the experimentally observed salt (ionic strength) effect on C3d–CR2 binding (19) (Morikis and Lambris, in preparation). Further studies also extend into modeling the contribution of non-polar, steric, and hydrogen-bonding interactions in the C3d–CR2 binding.

Our work in progress includes the design of theoretical mutations to allow us to determine the direct contribution of each of the experimental mutations on the C3d–CR2 interaction. We are also designing structures with multiple theoretical mutations that mimic the sequence of C3d from other species using human C3d as a structural template. Finally, we are designing structures of chimeric C3d molecules from evolutionary close and distant species that show differential binding to human CR2 (Morikis and Lambris, work in progress). Prior to the electrostatic calculations, these new structural designs will be subjected to molecular dynamics calculations and energy minimizations for optimization. Overall, our theoretical studies will be compared with existing and concurrent experimental work involving mutants, chimeras, and monoclonal antibodies (19,20). A detailed knowledge of the nature of C3d–CR2 interaction is expected to aid in drug design.

Mass Spectrometry and Hydrogen–Deuterium Exchange Studies

Hydrogen–deuterium exchange has traditionally been used to understand the formation of protein core or stable intermediate or

transient states in pathways of protein folding, because it provides a noninvasive method for identifying protected (or deprotected) exchanging amides. The same principles can be applied to studies of protein–protein association, where the loss in solvent-accessible surface area on association can be correlated with amide protection from exchange for the amides that lose their contact with solvent. Recent advances in the use of mass spectrometry allow for rapid collection of data of free and complexed proteins (21–23). Comparison of mass spectra of free and complexed proteins provides the sites of interaction without the need of previously available structural data. The efficiency and rapidity of the technique makes it a suitable method for the study of the multiplicity of interactions of complement components within the complement system and with other serum or membrane-bound proteins. We are currently conducting such studies, focusing primarily on the conformational changes of the C3 molecule, as well as its interaction with various ligands and receptors (e.g., C3d–CR2).

Rational Design of Complement Inhibitors: The Case of Compstatin

Complement activation is implicated in tissue injury in a variety of debilitating autoimmune and other diseases in pathological situations arising from bioincompatibility of human and artificial products, as well as in organ transplantation across species (24). As there are no clinically available drugs that inhibit complement activation, great research efforts are directed toward the discovery of complement inhibitors ranging from small molecules to large monoclonal antibodies (24,25,38). Our recent efforts have been focused on the identification of complement inhibitors that bind to C3, which is a complement component through which converge all

three pathways of complement activation—the classic, the lectin, and the alternative. We have used a phage-displayed combinatorial random peptide library to identify a 27-residue peptide that binds to C3 and inhibits complement activation (26). This peptide was truncated to a 13-residue cyclic segment that maintained complete activity, which we named compstatin. Compstatin inhibits the cleavage of C3 to C3a and C3b by C3 convertase. We have tested compstatin in a series of *in vitro*, *in vivo*, *ex vivo*, and *in vivo–ex vivo* interface experiments and found that compstatin: (1) inhibits complement activation in human serum (26), (2) inhibits heparin/protamine-induced complement activation in primates without significant side effects (27), (3) prolongs the lifetime of a porcine-to-human xenograft perfused with human blood (28–30), and (4) inhibits complement activation in models of cardiopulmonary bypass, plasmapheresis, and dialysis extracorporeal circuits (31). These and additional experiments (32) have also shown that compstatin has low toxicity. Based on these studies, and taking into consideration the small size of compstatin that allows for rapid and massive synthesis, it is apparent that compstatin is a good candidate to become a therapeutic agent.

The sequence of compstatin is ICVVQD-WGHRCT-NH₂, where Cys² and Cys¹² form a disulfide bridge. To understand the structural basis of the inhibitory activity of compstatin, we have determined its three-dimensional (3D) structure using homonuclear 2D NMR spectroscopy in combination with two separate experimentally restrained computational methodologies. The first methodology involved distance geometry, molecular dynamics, and simulated annealing (33), whereas the second methodology involved global optimization (34). The structure of compstatin revealed a molecular surface that

is composed of a polar patch and a nonpolar patch. The polar part includes a type I β -turn, and the nonpolar part includes the disulfide bridge. In addition, we synthesized and tested for activity a series of analogs with alanine replacements (an alanine scan) that revealed that the four residues of the β -turn and the disulfide bridge with the surrounding hydrophobic cluster are essential for inhibitory activity (33,35). Also, we have performed NMR studies on a series of compstatin analogs that were designed with the aim to enhance or disrupt the structure of the parent peptide (36). The NMR studies have revealed which individual residues contribute to the structural stability of compstatin, and parallel inhibitory activity studies have allowed us to make structure–activity correlations (36). We have also proposed a working model on the nature of association of compstatin with C3 based on the structure–activity and kinetic-binding studies (36,37).

We have used the collective knowledge of the biological and physicochemical characteristics of compstatin to design (1) linear analogs, (2) deletion analogs, (3) acetylated analogs at the N-termini, (4) conservative replacement analogs, (5) a retro-inverso analog, (6) analogs that promote β -turn formation, and (7) a second generation of phage-displayed analogs (26,36,38) (Soulika et al., submitted). We have identified several active compstatin analogs with improved activity in comparison to their parent peptide. In our continuing efforts to design more active compstatin analogs, we have established a fruitful collaboration with Professor Chris Floudas of Princeton University. Professor Floudas contributes a significant theoretical combinatorial component with global optimization approaches on structural information, which adds another level of rapid design in our phage-displayed-based combinatorial design and

rational structure–activity-based NMR design. Already, the theoretical combinatorial method has resulted in at least two analogs that are more active than their parent peptide (Klepeis et al., submitted). Finally, additional kinetic studies, some of which address the issue of species specificity of compstatin, have been performed (38a; Soulika et al, submitted).

Our NMR/computational studies are not limited to compstatin, but expand to other potential therapeutics and several components, or fragments of components, of the complement system and interacting proteins.

Proteomic Approaches for Studying Protein–Protein Interactions and “Profiling” Global Protein Expression

Proteomics is the study of all or a part of the gene transcripts in an organism or a given tissue sample (39). The basic principle is to separate a complex protein/peptide mixture into its components, then analyze quantitative changes or identify certain proteins of interest. The information about a cell’s proteome at a specific time-point can then be correlated with the genomic approach that has successfully been applied to the study of cell physiology in recent years. Furthermore, the proteome contains additional functional information about the investigated tissue or cells that cannot be retrieved from the corresponding genome or transcriptome (40).

Our laboratory applies different techniques in separating complex protein mixtures. They range from 2D electrophoresis to liquid chromatography and capillary electrophoresis (41). For the identification of the protein-mixture components, we utilize a MALDI-TOF mass spectrometer (MicroMass) after tryptic digestion of the isolated proteins. Every protein processed in that manner produces a unique

mass fingerprint (pattern of peptide fragment masses after tryptic digest) and can thereby be identified through searches of currently available protein databases. Second, we are able to obtain partial amino-acid sequence information from previously uncharacterized proteins by a second mass spectrometry (MS) step using an high performance liquid chromatography (HPLC) separation, followed by electrospray ionization MS (ThermoFinnigan). This involves further fragmentation of the primarily generated peptides.

For protein mixtures of higher complexity, our laboratory mainly applies 2D gel electrophoresis as the separation and quantification step. This process begins with the isoelectric focusing of the proteins, which separates them according to their isoelectric points, followed by a conventional electrophoretic analysis based on molecular size. Quantification of the proteins is then possible after staining and scanning (computer-based analysis, Image Master, Molecular Dynamics, UK). The proteins, which are detected as spots in the gels, are excised from the gel and subjected to tryptic digestion and MS.

We also perform conventional Western blots with antibodies against epitopes of interest on the 2D gels after membrane transfer. This gives us the opportunity to search for specific known proteins or differentiate multiple epitopes in complex protein mixtures.

We are currently applying the described methodology to investigate proteome changes in the context of complement C3 deficiency in mice. C3-deficient mice have widely been used to study various physiological functions of this key molecule of the complement system. From recent investigations, it has become evident that the complement system is not only involved in host-defense mechanisms, but can also exert a regulatory influence on many cells

and in a variety of physiological and pathophysiological situations (42–44).

Therefore, we have postulated that a large array of mechanisms may contribute to the phenotypic compensation observed in the C3-knockout mice. The liver, being the main source of the abundant serum protein C3, might display general proteome changes in animals lacking C3. These changes can either be evident under normal physiologic conditions or become detectable or even more prominent during increased hepatocellular metabolic demand.

To date, our results demonstrate significant changes in the liver proteome of quiescent complement C3-deficient animals after a 12-h fasting period in comparison to wild-type littermates. Five distinct proteins have been identified after tryptic digest and MS analysis and show significant differences in abundance in the quiescent liver of wild-type and deficient mice. The mechanism by which C3 influence their expression is under investigation.

These results imply that complement C3 deficiency has a substantial impact on the protein-expression profile of the liver. This influence goes beyond the lack of the specific gene product and demonstrates the value of including a proteomic approach in characterizing a gene-knockout model.

In an ongoing effort to expand our understanding of complement-mediated protein–protein interactions, our laboratory has integrated proteomic analysis in the study of C3–ligand interactions as a powerful analytical tool that will help us better define and monitor the complex and dynamic protein changes that underlie various complement-dependent biological responses. We are convinced that proteomics will become an indispensable tool in understanding complex biological systems by validating on a different level the large array

of genomic information that is already available in the databases.

The Complement System and Viral Molecular Mimicry

The complement system serves as both an innate and an acquired defense against viral infection. Activation of the C' system in the presence or absence of antibodies can lead to virus neutralization, phagocytosis of C3b-coated viral particles, lysis of infected cells, and generation of inflammatory and specific immune responses. To circumvent these defenses, viruses not only have developed mechanisms to control C' but have also turned these interactions to their own advantage. Our laboratory, in collaboration with Dr. H. Friedman (University of Pennsylvania, Philadelphia, PA), is actively engaged in a structural and functional analysis of viral proteins involved in C' evasion. The mechanisms developed by viruses to evade C' attack can be divided into three categories: (1) the structural and functional mimicry of human complement regulatory proteins, (2) the use of complement receptors for cellular entry and, (3) the use of human complement regulatory proteins. In our laboratory, we focus on the first two categories.

Structural and Functional Mimicry of Complement Regulatory Proteins as a Means of Evading Complement Attack

Sequence analysis of DNA viruses has revealed that some members of this group possess gene(s) with striking homology to the members of C' control proteins (CCP). Whether these viral genes have evolved from genes encoding regulators of complement activation (RCA) or from common ancestral genes, or whether they have been captured from the RCA loci of their hosts is not presently

known. The best known example of a structural homolog of the CCP is a vaccinia virus complement control protein (VCP). Other important C'-regulating viral proteins are glycoprotein C (gC) of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). These proteins do not show any structural similarity with CCP.

Structural Homologs

VCP is a 35-kDa secretory protein of vaccinia virus that contains four short consensus repeats (SCRs) (45). The protein shows homology to C4-binding protein (C4Bp) and many other members of the CCP group. Culture supernatant containing VCP and partially purified VCP has previously been shown to inhibit C'-mediated lysis of sheep erythrocytes, bind to C4b and C3b, and decay the classical and alternative pathway C3 convertases (46,47). Further studies have indicated that VCP acts as a cofactor in the proteolytic inactivation of C4b and C3b by factor I (47). However, factor I-mediated cleavages were not characterized in this study because the α' -chains of C3b and C4b had been degraded into small peptides, likely through the action of contaminating proteases.

To study and reevaluate the possible mechanisms by which VCP modulates the C' system, we have expressed VCP in a *Pichia* expression system. The expressed protein was purified to homogeneity and its biological activity was compared with those of human factor H and CR1. Recombinant VCP inhibited both the classical and the alternative pathway-mediated lysis of erythrocytes, although the concentrations were higher than those required for factor H and CR1. We noted that, in contrast to factor H and CR1, which display cofactor activity in factor I-mediated cleavage of C3b that results in the cleavage of the α' -chain at three different sites, rVCP primarily displayed cofactor activity for the first site (48). Thus, our results suggest that the interaction

of VCP with C3 is different from those of factor H and CR1 and that the single cleavage by factor I is sufficient to render C3b nonfunctional. To understand the kinetics of the interaction of VCP with C3b, we used Biacore technology. In this assay, we deposited C3b on the chip via its reactive thioester (49). This kinetic analysis revealed that VCP reacts with C3b, but not C3d. To further understand the mechanisms of complement inactivation by VCP, we have developed monoclonal antibodies (MAbs) that react with VCP (E. Argyropoulos et al., submitted). We used the recombinant VCP expressed in *Pichia* to vaccinate mice for the development of VCP-specific hybridomas. Hybridomas expressing MAbs that recognized VCP were selected and cloned. We have begun the characterization of 10 VCP-specific MAbs. All 10 MAbs recognized VCP on Western blot under reducing conditions, as well as native-bound VCP in a sandwich ELISA. Using an ELISA-based assay to determine if any of the MAbs block the interaction of VCP with C3b, we found MAbs that block the interaction of VCP with C3b and others that recognize VCP bound to C3b. We also hope to use these MAbs to further characterize the contribution of VCP in pathogenesis. VCP has important in vitro and in vivo effects. We showed that incubation of virions (in the absence of VCP) with anti-vaccinia antibody and complement results in decreased infectivity of vaccinia virus in tissue culture (50). The presence of VCP completely abrogates the antibody-dependent, complement-enhanced neutralization of vaccinia virions. Animal studies comparing wild-type virus to a mutant vaccinia virus lacking VCP showed that the mutant virus was attenuated. This effect in vivo is likely due to enhanced complement-mediated neutralization of virus lacking expression of VCP (50). Thus, VCP is a protein encoded by vaccinia virus that interferes with the host immune defense.

Proteins that have four SCRs and are also homologous to C' control proteins have been found in herpesvirus saimiri (HVS) and the recently discovered Kaposi's sarcoma-associated herpesvirus (HHV-8). These proteins are approx 30% identical in sequence with the human C' control proteins and with each other. Sequence analysis of cDNAs has shown that transcripts of the C' control protein of HSV (termed CCPH) exist as unspliced or single-spliced mRNAs. The unspliced species of mRNA directs the translation of a membrane-bound glycoprotein (mCCPH), whereas the spliced species encode for a secretory protein. In addition to CCPH, HVS also contains a gene that directs the expression of a homolog of CD59 named HVSCD59 (51). Currently, in collaboration with Dr. A. Sahu (National Center for Cell Science, Pune, India), we are expressing CCPH of HVS and HHV-8 to study and compare the mechanisms of C' inactivation by these novel proteins.

Other Viral Proteins that Regulate Complement

Viruses have also adopted proteins that are structurally different from CCP, but also function to inactivate complement. Important examples are gC molecules of HSV-1 and HSV-2, which are expressed on the virion envelope and on the surface of infected cells (52). Studies have clearly indicated that gCs regulate C' and provide protection against C'-mediated viral neutralization (53,54). gC of HSV-1 (gC-1) and HSV-2 (gC-2) are highly glycosylated proteins with numerous O-linked oligosaccharides and nine and seven potential sites for N-linked glycosylation, respectively (55). Both proteins bind to C3b when they are expressed on the surface of transfected cells or in the form of purified proteins (56). Previous reports have suggested that gC-1, like other regulators of C' activation (factor H, CR1, and DAF), accelerates the decay of a

bimolecular C3 convertase (C3b,Bb) into its subunits; however, in contrast to factor H and CR1, it does not mediate the proteolytic inactivation of C3b by factor I (57). In collaboration with Drs. Cohen, Eisenberg, and Friedman (University of Pennsylvania), we have shown that gC-1, but not gC-2, can destabilize C3 convertase by inhibiting the binding of properdin to C3b (58). We have also shown that, unlike other regulators of C' activation (factor H, MCP, DAF, and CR1), which interact with C3b, both gC-1 and gC-2 bind to native C3 (59). Furthermore, we have shown that the binding sites for gC-1 and gC-2 are located in the C3c region of C3, and that the C3 region spanning residues 727–768, which is implicated in the binding of CR1 to C3b, is not important for the gC–C3 interaction. Thus, gC-1 does not act as an analog of CR1. On the basis of the data obtained thus far, we propose that the activation of C' is not necessary for gC–C3 interaction, and that gC-1 can inhibit C' activity by blocking the binding of properdin and C5 to C3b. Complement-mediated neutralization of herpes virions occurs via a C5-dependent mechanism that does not require viral lysis, aggregation, or blocking virus attachment (60). Furthermore, in collaboration with Dr. Harvey Friedman, we have also helped show that gC–C3 interactions are involved in pathogenesis (61,62).

Use of Complement Receptors for Cellular Entry

Several viruses have developed strategies that allow them not only to evade C' attack, but also to use complement receptors for cellular entry (63). One of the important and well studied example of this group is the Epstein–Barr virus (EBV), which infects B cells through CR2 (64). Some T cells and dendritic cells also express this receptor (65). CR2 binds to iC3b, C3dg, and C3d fragments of C3 (66) and shows a low-affinity

binding to C3b (67). In collaboration with Dr. Constantine Tsoukas (San Diego State University), we have identified a novel EBV receptor on HSB-2 cells, which is unrelated to CR2. This receptor binds to EBV and C3, but not to anti-CR2 antibodies (68). Detailed biochemical characterization of this receptor is in progress. Other viruses that use C' receptors for cellular entry include measles, echo, and West Nile viruses. These viruses use membrane cofactor protein, decay acceleration factor, and CR3, respectively, for initiating infection (63,69,70).

Complement Evolution and Diversity

Studies of evolutionary processes that give rise to living organisms as they are known today constitute an integral part of modern biology. A comprehensive understanding of any complex biological system, such as the complement system, requires a thorough knowledge of its origin and evolution, development, and diversity. For this reason, our laboratory has been engaged in the study of the evolution of the complement system for the last 15 yr.

Invertebrate species have fewer complement components and possess only the alternative and lectin complement activation pathways (71). Although complement-like activity has been detected in a variety of invertebrates (72), the sea urchin (echinoderm phylum) is the most ancient species from which a complement-like component with a high-sequence similarity to gnathostome C3 has been purified (73). We have been able to isolate this sea urchin molecule in collaboration with Dr. L. Courtney Smith (George Washington University, Washington, DC), who has obtained its primary sequence. The presence of this molecule in these organisms suggest that the C' system is a very ancient

immune mechanism (at least 600–700 million years old), predating the emergence of the vertebrates.

In our efforts to characterize the C3 molecules of invertebrates and lower vertebrates, we detected the existence of multiple C3 isoforms in two teleost fish species: the rainbow trout, *Oncorhynchus mykiss*, and the gilthead seabream, *Sparus aurata* (74,75). In all other animals studied thus far, C3 was shown to exist as a single-gene product. The three trout C3 isoforms (C3-1, C3-3, C3-4) are products of different genes and significantly differ in their binding efficiencies to various C'-activating surfaces (74). Five C3 variants (C3-1, C3-2, C3-3, C3-4, and C3-5) have been isolated from the sea bream. The observed structural and functional diversity of C3 in teleost fish might serve to augment the number of potential pathogens that these C3 isoforms can recognize. We have hypothesized that teleost fish have developed a unique approach to expand their innate immunity by duplicating their C3 genes. This strategy might reinforce their immune response to foreign pathogens, because the specific immune response in fish is relatively weak in comparison to that of mammals. Thus, the structural and functional C3 diversity observed in fish may have important consequences for our understanding of the evolution of the C3 molecule, as well as innate immune processes.

In collaboration with Dr. Maria Rosario Pinto (Laboratory of Cell Biology, Naples, Italy), we recently demonstrated the presence of two C3-like genes in the invertebrate urochordate *Ciona intestinalis* (76). We indicated the presence of their transcription products in blood cells and the encoded proteins in the body fluid. This study provides further insight into the evolutionary origin of the ancestral molecules that gave rise to the different members of the $\alpha 2$ macroglobulin family of thiol-

ester-containing proteins (C3, C4, α 2 macroglobulin).

The earliest species from which lytic C' components have been identified are the cartilaginous fish (e.g., sharks) (77). These animals are also the first to possess an adaptive immune system and the classical pathway of complement activation. Although all three C' activation pathways have been shown to be present in cartilaginous fish, the nature and similarities of the complement molecules are still ill-defined. Recently, a C6-like molecule has been identified in the invertebrate cephalochordate, the amphioxus (78). This finding suggests that the lytic pathway may have a more ancient origin than previously believed. Teleost fish express all lytic complement components and are able to kill microorganisms by cytolysis. We have purified C5 from the trout and gilthead seabream and cloned the trout C5 gene (79). In addition, we have cloned the genes for C7 and C8 β of trout (A. Kazantzi et al., in press). The identification and characterization of these molecules will help us shed light on the evolution of the lytic pathway and the importance of cytolysis as an immune-effector mechanism.

An important function of the C' system, which appeared to have evolved early, is that of opsonization: the tagging of foreign particles for destruction by phagocytosis. A CR3 or CR4-like molecule has been identified in the invertebrate urochordate *Halocynthia roretzi* (80). Like mammalian CR3 and CR4, the molecule appears to be an integrin composed of an α and a β chain, and an antibody against the α subunit-inhibited C3-dependent phagocytosis of yeast cells by hemocytes.

We are currently focusing on the identification and characterization of CRs in fish. Thus far, no CRs have been identified in this group of animals, but the presence of a CR3-like molecule in urochordates strongly suggests the

presence of a similar type of receptor in fish. Mammalian CRs differ in their binding affinity to the various C' degradation products. We have purified and expressed several potential ligands of the CRs in trout. The C3b fragments of the three trout C3 isoforms were generated from intact C3 by in vitro cleavage with purified trout factor B and D (81). In mammals, C3b displays a high affinity for CR1. The ligand for CR3 is iC3b, and we have purified an iC3b-like fragment from trout serum. The C3d fragment, which exhibits a high affinity for the CR2 in mammals, has been expressed in *E. coli* for all three trout C3 isoforms. In addition, the ligands for the anaphylatoxin receptors, C3a and C5a, have been expressed. Over the years, we have generated a wide array of poly- and monoclonal antibodies that recognize distinct epitopes on the C3 molecule (82). The availability of these antibodies allows us to study the fragmentation pattern of C3 molecules after C' activation. The above described ligands are currently being used to locate binding sites on trout leukocytes, using freshly obtained cells from live individuals as well as from characterized cell lines.

Our laboratory, in collaboration with Dr. Ioannis Zarkadis (University of Patras, Greece), has also been involved in the characterization of complement regulatory molecules from teleost fish (rainbow trout). The main function of these proteins, which are either soluble or membrane-bound, is the protection of host cells from autologous complement activation and tissue injury. Factor H is the only member of the family of complement regulators that has been cloned and characterized in lower vertebrates (teleost fish) (83). SBP1, a protein with factor H- and C4bp-like activity, has been purified and cloned from the sand bass *Paralabrax nebulifer*. Factor H-like clones have been recently isolated from the rainbow trout, and sequence analysis has

revealed high similarity to the factor-H homolog SBP1 isolated from the sand bass (Zarkadis et al., unpublished data). The potential function of such factor H-like molecules in trout is currently under investigation.

Because teleost fish appear to represent a critical point of divergence in the evolution of the C' system and present an array of C' proteins with unusual functional properties, these animals constitute a singularly useful model for analyzing the origin, development, and structural and functional diversity of this immune defense system at the molecular level. In addition, the knowledge obtained should also contribute to solving health-related problems in farmed fish.

Complement Components Mediate Novel Functions in Developmental Processes

Complement has long been recognized as an arm of innate immunity that mediates strictly immunologic functions by maintaining host defense against invading pathogens and by mediating local and systemic inflammatory responses under various pathophysiological settings. Recently, however, it has become evident that several complement components exert novel functions that are associated with normal biologic processes in various tissues and cannot be clearly placed in an "inflammatory" context. These nontraditional functions of complement, which have remained rather elusive from the broader scientific community, clearly indicate that complement, a phylogenetically ancient system of immunity, has retained functional links with other cellular networks that influence normal developmental pathways.

Over the past 5 yr, our laboratory has been actively engaged in studies to delineate the role of various complement components in three distinct developmental processes: limb and lens regeneration in urodeles, liver regen-

eration in mammals, and stem cell differentiation during hematopoietic development. This work has been made possible through the ongoing collaboration with Drs. Panagiotis A. Tsonis (University of Dayton, OH) Katia Del Rio Tsonis (University of Miami, FL) (urodele regeneration studies), and Dr. Mariusz Z. Ratajczak (University of Louisville, KY) (role of complement in hematopoiesis).

The Role of Complement in Liver Regeneration

The liver is one of the few organs in mammals that have retained the ability to regenerate, restoring its functional integrity after partial hepatectomy and viral or acute toxic injury (84). The liver parenchyma reacts to these insults by eliciting a robust proliferative response that causes previously quiescent hepatocytes to reenter the cell cycle and divide. Several cytokine, hormonal, and growth factor-dependent pathways have been implicated in triggering liver regeneration (85–87). However, the wide array of mechanisms and factors that act cooperatively to induce the mitogenic response of hepatocytes still remains to be elucidated.

In an effort to dissect the involvement of different complement proteins in mammalian regeneration, we have assessed the regenerative response of specific complement-deficient mouse strains in two well-established models of liver regeneration: carbon-tetrachloride-mediated acute liver injury and partial hepatectomy.

We originally showed that C5-deficient mice have defective liver regeneration after CCl₄-mediated liver injury (42). Furthermore, a critical role for the C5a anaphylatoxin and its receptor C5aR in liver regeneration was demonstrated by the severely delayed entry of hepatocytes into S-phase after pretreatment of CCl₄-injured mice with a specific C5aR antag-

onist (42,88). To further elucidate the role of complement in liver regeneration, and to dissect the contribution of C5aR-mediated pathways in the regenerative response of the liver, we then investigated the regenerative capacity of mice treated with a specific C5aR antagonist in a partial hepatectomy model.

Blockade of the C5aR using the specific antagonist AcF{OpdChaWR} resulted in the impaired liver regeneration and extensive parenchymal necrosis at 44 h after partial hepatectomy. This defective regenerative response was underlined by the diminished BrdU incorporation in hepatocytes (>90% reduction in BrdU uptake) and the concomitant absence of mitotic figures throughout the liver parenchyma of the antagonist-treated mice. In contrast to the antagonist-treated mice, the livers of control (scrambled peptide-treated) mice manifested a normal regenerative course with vigorous regeneration throughout the parenchyma, absence of hepatocellular necrosis, and pronounced BrdU incorporation in hepatocytes. In further support of these data, pretreatment of mice with an affinity-purified a-mouseC5aR antibody generated in our laboratory, resulted in attenuated liver regeneration with markedly depressed hepatocyte proliferation (approx 75% reduction in BrdU labeling in comparison to control IgG-treated livers) and extensive parenchymal necrosis at 44 h after liver resection.

Similarly, C3-deficient mice show impaired liver regeneration after partial hepatectomy with a high-mortality rate in comparison to their wild-type counterparts. Reconstitution of these mice with a single dose of C3 prior to surgery effectively restored their regenerative response to wild-type levels. The mechanism by which C3 is involved in this process is currently being addressed, using a combination of *in vivo* reconstitution and blockade studies.

Preliminary studies using semiquantitative RT-PCR approaches have shown that C5aR mRNA expression is markedly upregulated in the liver within 6 h after partial hepatectomy. The functional implications of this C5aR induction include a possible interaction with local cytokine-driven pathways. The potential role of C5aR in modulating intrahepatic and systemic cytokine responses during liver regeneration is currently under investigation. Furthermore, the global effect of C5aR stimulation on the transcriptional activation of cell-cycle related genes in hepatocytes is being characterized by gene-expression profiling approaches (e.g., cDNA microarrays).

Taken together, our studies clearly indicate that abrogation of C5aR signaling (either by genetic deletion of upstream mediators, such as C3-C5, or by receptor-targeted inhibition using peptide antagonists) contributes to impaired liver regeneration after partial hepatectomy or acute liver injury. Delineating the mechanisms by which complement proteins and receptors interact with other signaling networks in the regenerating liver will provide further insight into the molecular pathways that drive the early growth response of the liver and “prime” quiescent hepatocytes to reenter the cell cycle and proliferate.

In an effort to broaden our understanding of the dynamic nature of liver regeneration and elucidate the complex protein-protein interactions that complement modulates in the hepatic interface, we have recently initiated studies of global protein expression in the regenerating liver by employing proteomic approaches. Using C3-deficient mice, and applying the model of partial hepatectomy, we have been able to show that the lack of C3 protein is associated with distinct changes in hepatic protein expression after partial hepatectomy (C. Strey et al., unpublished observations). In this context, we have identified at

least five proteins in the liver of heptec-tomized C3-deficient mice that show quanti-tative differences on the basis of 2D gel data analysis in comparison to wild-type litter-mates. These studies are in progress and will essentially complement our research on the role of complement in liver regeneration.

Complement and Urodele Regeneration

The ability to regenerate complex structures and reconstruct entire body parts from dam- aged tissues is a trait widely encountered among invertebrates (e.g., annelids, hydroids, and so forth) and in lower vertebrates, such as amphibians (89). In urodele amphibians (axolotls), the process of regeneration is quite prominent in the limb and tail and in struc- tures of the eye (retinal epithelium and lens) (90,91). Limb regeneration in urodeles entails the activation of complex developmental pathways that act in concert to promote the dedifferentiation, proliferation, and rediffer- entiation of mesenchymal cells into the spe- cific cell types that comprise the various tissues of the regenerating limb. A morphogenetic hallmark in the process of limb regeneration is the formation of the blastema, a cell layer consisting of dedifferentiated epithelial and mesodermal cells that gradually receive sig- nals from the local microenvironment (e.g., wound epithelium), reenter the cell cycle, and undergo differentiation into the various cell types that will reconstitute the muscle, bone, and connective tissue of the regenerat- ing limb.

The molecular pathway(s) that underlie these phenotypic transitions and develop- mental stages are largely unknown. In a previous study, we have shown that the com- plement component C3 is expressed in the blastema cells located in the regenerative zone of the amputated limb (92). Interestingly, C3 was also detected in blastema cell cultures of

the myogenic lineage, a finding which sug- gests that C3 may be critical to muscle devel- opment and myoblast differentiation in settings that evoke extensive muscle reconstruction, such as limb regeneration.

The distinct expression pattern of C3 in tis- sues that undergo extensive remodeling during limb regeneration (e.g., muscle) has led to the speculation that complement proteins may serve as important mediators of tissue regen- eration, in part by promoting cell–cell inter- actions that are critical to matrix degradation and tissue remodeling.

To further study the effect of complement in urodele regeneration and to dissect the specific involvement of the critical compo- nents C3 and C5 in limb and lens regenera- tion, we chose the newt (*Notophthalmus viridescens*) as a model organism that pos- sesses extensive regenerative capacity in both these tissues. To study the expression during newt lens and limb regeneration, we have iso- lated newt cDNAs for complement C3 and C5 and have generated antibodies against C3a and C5a molecules; these antibodies have been shown to be specific for C3 and C5, respec- tively, and have been found to inhibit their activation (Y. Kimura et al., submitted). Expression of both proteins was demonstrated in limb and lens structures during regenera- tion by immunostaining using the respec- tive polyclonal antibodies. The expression of C3 and C5 was also confirmed by *in situ* hybridization.

To assess the *in vivo* role of complement in regeneration, cobra venom factor was injected into newts before amputation and was found to cause a significant delay in limb regenera- tion. In contrast, similar treatment before len- tectomy resulted in bigger fiber formation in the lens. To dissect the role of C3 and C5 in regeneration, we have injected newts with anti- C5a or anti-C3a antibodies before amputation

or lentectomy, and we are in the process of analyzing their effect in both limb and lens regeneration.

A Role for Complement in Early Hematopoietic Development

The role of various complement regulatory molecules and receptors in protecting blood cells from complement-mediated lysis and promoting their inflammatory recruitment and activation during the course of infection (93) is well-appreciated. However, very little is known about the distribution of complement components in early hematopoietic progenitor cells, their potential role in the commitment of different precursors to various lineages during embryonic development, and the complement-mediated interactions that influence the homing of lymphoid progenitors in various tissues.

Recent studies suggest an intriguing role for the phagocytic complement C1q receptor (C1qRp) in early hematopoietic development (94,95). In this respect, a novel fetal stem cell antigen (AA4) was recently identified as the mouse homolog of human C1qRp (94).

Our laboratory, in collaboration with Dr. Mariusz Ratajczak (University of Louisville, KY), has been involved in a study profiling the expression of various complement components and receptors in normal human early stem/progenitor cells, as well as in lineage-committed hematopoietic cells. We were able to show that the GPCRs for both C3a and C5a anaphylatoxins are expressed by human clonogenic CD34⁺ cells, and that both complement components C3 and C5 are secreted by the bone marrow stroma (R. Reca et al., submitted). Furthermore, there is evidence suggesting that the C3a anaphylatoxin plays a role in the maturation and lineage commitment of hematopoietic progenitors, because in serum-free cultures, it was found to costimulate the

development of cells within the erythroid and megakaryocytic, but not granulomonocytic, lineages (R. Reca et al., submitted).

In addition, stimulation of the C3a receptor (C3aR) appears to regulate the chemotaxis of human CD34⁺ cells by synergistically increasing the migration of these cells in the presence of α -chemokine stromal-derived factor-1 (SDF-1)(96) (R. Reca et al., submitted).

The striking observations that C3, like SDF-1, is secreted by bone marrow-derived stromal cells, and that both C3aR and CXCR4 are expressed by human CD34⁺ cells, have laid the groundwork for further investigation of the hypothesis that a functional crosstalk between the C3aR and CXCR4 signaling pathways may play an important role in the homing of human stem/progenitor cells to the bone marrow hematopoietic niches.

Perspectives

It has been 37 yr since the first isolation and characterization of the C3 molecule. During this exciting era of complement research, a wealth of information has been generated on the C3 structure and its role in complement activation and immune response pathways. Nevertheless, a complete picture of the C3 interaction with its ligands has not yet emerged, in part because the 3D structure of the molecule is still unavailable. In the past, a variety of approaches, including the generation of synthetic peptides corresponding to various regions of C3, development of monoclonal and site-specific antipeptide antibodies against C3, site-directed mutagenesis, and expression of chimeric molecules, have been used to define the binding sites on C3. Each of these methods has its limitations. We believe that conclusions concerning the involvement of a particular region in ligand binding can be drawn only if all of the above methods point

in a single direction. Therefore, identification of the functional domains of C3 by the methods discussed above, together with determination of 3D crystallographic structures or NMR-derived solution structures, is needed if we are to give a complete understanding of C3–ligand interactions. This information will not only help us in understanding the overall structure–function relationships exhibited by the molecule, but will also assist us in designing better C′ inhibitors and further our knowledge of the interactions between C3 and proteins of foreign origin (e.g., viral proteins). Finally, elucidating the evolution of the molecule promises to enhance our understanding

of the structural features that are critical for its function in addition to its evolution. It is our conviction that studies of the evolution of C3 will reveal many unexpected and significant features of C3 that will expand our understanding of the C′ system and lead to improved treatment of C′-related disease.

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