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Review

From atoms to systems: a cross-disciplinary approach to complement-mediated functions☆

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Abstract

With an ever-increasing wealth of information made available to researchers from expanding genomic sequence and protein structure databases, traditional experimentation and research are being drastically revisited. The unidirectional study of single molecules and pathways is being replaced by a combinatorial and cross-disciplinary platform that investigates interactive biological systems and dynamic networks. The complement system constitutes an ideal paradigm of how this concept is being applied in the field of contemporary immunology. Our laboratory has adopted such a cross-disciplinary approach in elucidating key aspects of complement functions and determining the role of several complement proteins in both inflammatory and developmental processes. Here we discuss recent findings pertaining to the rational development of complement inhibitors, our studies on protein–protein interactions and our progress in the study of viral immune evasion and complement evolution. Furthermore, we present recent studies implicating complement components in complex developmental processes, such as organ regeneration, hematopoietic development, and stem cell engraftment.

Keywords: Complement; Evolution; Regeneration; Compstatin; Therapeutics; Vaccinia; Mass spectrometry; Proteomics

1. Introduction

In an era that nurtures the integrated study of biological systems as the prevalent concept in contemporary scientific thinking, complement research is being revisited and our current knowledge of this innate immune system is enriched by findings that point to novel functions that do not strictly correlate with immunological defense and surveillance, immune modulation or inflammation (Mastellos and Lambris, 2002).

Indeed, as the Greek philosopher Heracleitus once claimed, "Nature is fond of hiding itself" and this is becoming even more evident in the light of fascinating research that suggests that complement proteins exert functions that are either more complex than previously thought or go well beyond the innate immune role of the system.

Departing from traditional hallmarks of molecular biology such as the genome and the transcriptome, and beginning to appreciate more the "proteome" as the dynamic expression profile and unique 'fingerprint' of all organisms, novel associations between biochemical pathways and apparently unrelated biological processes are constantly revealed. In this respect, recent evidence produced by our laboratory (and others) suggests that complement components can modulate diverse biological processes by closely interacting with other intra- and intercellular networks (Mastellos and Lambris, 2002).

Furthermore, the structure and functions of several complement proteins as well as the protein–protein interactions that underlie these functions are now being investigated with the aid of cross-disciplinary approaches ranging from mathematics and biophysics to comparative phylogenesis, molecular modeling, mimetics and proteomics. Our laboratory in collaboration with others, extending its research beyond the scope of traditional complement pathobiology, has embraced this global and combinatorial approach to biomedical research and has been actively engaged in defining the function of complement proteins in several biological contexts and pathophysiological states.

The complement system has been long appreciated as a major effector arm of the innate immune response. It consists of a complex group of serum proteins and glycoproteins and soluble or membrane-bound receptors, which play an important role in host defense against infection (Lambris, 1988). Complement, a phylogenetically conserved arm of

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innate immunity, functions together with the adaptive immune response by serving as an important inflammatory mediator of antigen–antibody interactions. 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 (Sahu and Lambris, 2001).

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 interaction of complement components with specific carbohydrate groups and lipopolysaccharides present on the surface of foreign pathogens (e.g. bacteria) (Muller-Eberhard, 1988). 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. During the course of this cascade, a number of biological processes are initiated by the various complement components, including inflammation, leukocyte migration, and phagocytosis of complement-opsonized particles and cells. The end result of these complement-mediated events is a direct lysis of target cells and microorganisms as a consequence of membrane-penetrating lesions (pores). Currently over 30 complement proteins have been identified, and deficiencies in any particular components have been frequently associated with a diminished ability to clear circulating immune complexes or fight infection.

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.

2. Studies on protein–protein interactions of complement components

2.1. Immunophysics: computational studies of protein–protein interactions in the complement system

Computer simulation is a quantitative tool to predict and model biomolecular structure, dynamics, and interactions. Computational studies are based on algorithms that represent physical models of important biological processes and take into account the underlying physics and chemistry of the biomolecular building blocks, both microscopically and macroscopically. Computational studies at atomic level require the availability of three-dimensional structures from crystallographic or NMR studies that provide the molecular detail of the individual elements of the protein, as well as the properties of the surfaces and volume of the protein as a whole. Increasing numbers of structures of protein complexes are being deposited at the protein data bank. Comparison of the structure of the complex with the free structures of its components allows for insight into the structural changes occurring upon 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 non-polar side chains, hydrogen bonding interactions, electrostatic interactions of ion pairs (salt bridges), and steric (van der Waals) interactions. Additionally, electrostatic complementarity and shape complementarity of the molecular surfaces and volumes 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 to understand the bio-physico-chemical nature of the interactions that involve complement components. The nature of the interaction between complement components iC3b or C3d with complement receptor type-2 (CR2) has been a subject of intense scrutiny for a long time and by several groups. Experimental studies have shown that this interaction depends strongly on the solution pH and ionic strength (Moore et al., 1989; Diefenbach and Isenman, 1995; Clemenza and Isenman, 2000; Guthridge et al., 2001; Sarrias et al., 2001), an observation that is indicative of the involvement of electrostatics in the association. The experimental data have prompted us to perform electrostatic calculations to understand the nature of C3d-CR2 association at a theoretical level. The electrostatic calculations are based on the solution of the finite difference linearized Poisson–Boltzmann equation with continuous solvent representation and proper atomic charge and van der Waals radius parametrization. The calculations were made possible using the atomic detail of the three-dimensional structures of human C3d (Nagar et al., 1998), CR2 (SCR1–2) (Prota et al., 2002), and C3d–CR2 (SCR1–2) complex (Szakonyi et al., 2001), which are available from crystallographic data. The structure of the complex of C3d–CR2 (SCR1–2) has revealed the sites of association of the two molecules, which are dominated by the presence of hydrophobic and steric interactions, as well as hydrogen bonds involving mainly backbone atoms on the side of C3d. These are not sufficient to explain the observed pH and ionic strength dependence of C3d–CR2 association. In addition, in association studies between iC3b and cell-bound full-length CR2, site-directed mutations of single or up to three charged residues within the C3d region of iC3b were responsible for modulating the binding ability of iC3b with CR2 (Clemenza and Isenman, 2000). Mutations of specific acidic residues resulted to loss (even elimination for some) of binding ability; on contrary, a single mutation of a basic residue resulted to doubling of the binding ability. However, this study involved mutations of residues that are not located at the association

interface of C3d with CR2 (SCR1–2), which was revealed by the crystallographic structure of the complex (Szakonyi et al., 2001). Taking into account that only the first two modules of CR2 (out of a total of 15 or 16) are responsible for association with C3d (Lowell et al., 1989; Carel et al., 1990; Kalli et al., 1991), the mutagenesis, pH, and ionic strength experimental data cannot be explained by electrostatic interactions among charged residues located at the association interface alone; charged residues away from the association interface should be contributing to association.

Following our physical intuition we performed electrostatic calculations on C3d and CR2 (SCR1–2) in their free and bound forms. Our goals were, first to explore the involvement of the overall electrostatic field of each protein in binding, and second to measure the contribution to binding for each ionizable (charge carrying depending on pH and apparent pK_a) residue within each protein. The calculation of electrostatic potentials revealed that free C3d is predominantly negatively charged and free CR2 (SCR1–2) is predominantly positively charged (Morikis and Lambris, 2004). Indeed, C3d and CR2 (SCR1–2) are macro-dipoles with excess of negative and positive total charge, respectively. The same applies on the C3d–CR2 (SCR1–2) complex, which has a more balanced macro-dipole character. Theoretical site directed mutations were constructed (Morikis and Lambris, 2004) on the crystallographic structures to address the experimental site-directed mutagenesis data (Clemenza and Isenman, 2000). The calculated electrostatic potentials using the theoretically mutated C3d structures showed modulation that correlated well with the experimental data. This led us to propose a two-step association model comprising recognition and binding (Morikis and Lambris, 2004). According to this model the overall electrostatic fields of C3d and CR2 (SCR1–2) are responsible for recognition and acceleration of binding compared to diffusional encounters, while specific interactions of side chains within the association interface are responsible for securing binding. The latter interactions involve the whole range of hydrophobic, van der Waals, hydrogen bonding, electrostatic, and favorable shape complementarity. In addition, the pairwise electrostatic interactions among all ionizable residues of C3d, CR2 (SCR1–2) and C3d–CR2 (SCR1–2) have been determined and apparent pK_a values for every ionizable residue have been predicted (Morikis and Lambris, 2004). The latter provide a quantity that can be easily interpreted both theoretically and experimentally. Apparent pK_a values also contribute to our detailed understanding of the role of each ionizable residue in building up networks of electrostatic interactions. A detailed knowledge of the nature of C3d–CR2 interaction is expected to aid in drug design.

2.2. 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 non-invasive method for identifying protected (or de-protected) exchanging amides. The same principles can be applied to studies of protein–protein association, where the loss in solvent-accessible surface area upon 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 (Smith et al., 1997; Mandell et al., 1998a,b). 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).

2.3. Rational and combinatorial design of complement inhibitors

2.3.1. 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 bio-incompatibility of human and artificial products, and in organ transplantation across species (Sahu and Lambris, 2000). As there are no clinically available drugs that inhibit complement activation, great research efforts are directed towards the discovery of complement inhibitors ranging from small molecules to large monoclonal antibodies (Lambris and Holers, 2000; Sahu and Lambris, 2000). Our recent efforts have been focused on the identification of complement inhibitors that bind to complement component C3. All three complement activation pathways, the classic, the lectin, and the alternative, converge to C3, which is also the starting point of the common pathway.

A phage-displayed combinatorial random peptide library was used to identify a 27-residue peptide that binds to C3 and inhibits complement activation (Sahu et al., 1996). This peptide was truncated to a 13-residue cyclic segment that maintained complete activity, which was named compstatin. Complement activation beyond C3 is stopped by binding of compstatin, which hinders the cleavage of C3 to pro-inflammatory peptide C3a and opsonin C3b by C3 convertases. The function of compstatin has been tested in a series of in vitro, in vivo, ex vivo, and in vivo/ex vivo interface studies, which found that compstatin: (1) inhibits complement activation in human serum (Sahu et al., 1996), (2) prevents heparin/protamine-induced complement activation in primates without significant side effects (Soulika et al., 2000), (3) inhibits complement activation in models of extra-corporeal circuits for cardio-pulmonary bypass

surgery, plasmapheresis, and dialysis (Nilsson et al., 1998), (4) prolongs the lifetime of a porcine-to-human xenograft perfused with human blood, in a xenotransplantation model (Fiane et al., 1999a,b; Fiane et al., 2000), (5) blocks the E. coli-induced oxidative burst of granulocytes and monocytes (Mollnes et al., 2002), (6) inhibits complement activation by cell line SH-SY5Y (Klegeris et al., 2002), (7) shows low toxicity or no adverse effects when these were studied (Furlong et al., 2000), and (8) shows species-specificity, being active with primate C3 but not with C3 from lower species (Sahu et al., 2003). The functional and toxicity studies of compstatin, together with its small size that allows for rapid and massive synthesis, suggest that compstatin may be a promising candidate for optimization aiming to the development of a therapeutic agent.

The sequence of compstatin is I[CVVQDWGHHRC]T-NH_2, where the brackets denote the formation of disulfide bridge between Cys2 and Cys12. To understand the structural basis of the inhibitory activity of compstatin that allows for the design of analogs with increased inhibitory activity, we performed homonuclear 2D NMR spectroscopy. It is known that small peptides in solution form ensembles of dynamically inter-converting conformers. This is the case of compstatin for which the NMR observables have shown conformational averaging. The structure of a major conformer of compstatin was determined using NMR-derived restraints and two separate computational methodologies. The first methodology involved restrained distance geometry, molecular dynamics, and simulated annealing (Morikis et al., 1998) and the second methodology involved restrained global optimization (Klepeis et al., 1999). The structure of compstatin revealed a molecular surface that comprises of a polar patch and a non-polar patch. The polar part (residues 5–11) includes a Type I β-turn and the non-polar part (residues 1–4 and 12–13) includes the disulfide bridge that cyclizes the peptide. 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 (Morikis et al., 1998, 1999).

The collective knowledge of the biological, structural, and physico-chemical characteristics of compstatin was used as a basis for several rounds of sequence, structure, and binding/activity optimization. The amino acid physico-chemical properties that are responsible for residue–residue interactions, which contribute to structure formation, binding, and activity are hydrophobicity/polarity, charge, van der Waals interaction, potentiality for hydrogen bond formation, and electronic distribution. The propensities of amino acids for specific secondary structure formation were also considered, in combination with topological and dynamic properties such as side chain length, volume and branching, side chain orientation and mobility, isomerism, and backbone flexibility.

The first round of rational design identified that acetylation of the amino terminal residue, Ile1, produced a three-fold more active analog than the parent peptide (Sahu et al., 2000; Furlong et al., 2000). We attributed this increase to the removal of the disruptive positive amino-terminal charge from the vicinity of the hydrophobic cluster (Morikis and Lambris, 2002; Morikis et al., 2002; Soulika et al., 2003).

The second round of rational design identified a four-fold more active analog, with sequence Ac-I[CVVQDWGA-HRC]T-NH_2 (Morikis et al., 2002). In this round, NMR studies were performed on a series of compstatin analogs that were designed to perturb the major structural characteristics of compstatin, which are the hydrophobic cluster, the β-turn, and the disulfide bridge. The perturbation involved local structure enhancement or disruption or introduction of flexibility. The NMR studies revealed which individual residues contribute to the structural stability of compstatin and parallel inhibitory activity studies allowed us to make structure–activity correlations. Fine-tuning of the structural properties and inhibitory activities was accomplished by conservative residue replacements that maintained the hydrophobic or polar or charged amino acid character (Morikis et al., 2002).

The first two rounds of rational design also included the design of linear analogs, deletion analogs, an unblocked analog at the termini, analogs with alternative terminal residues, radical replacement analogs, a retro-inverso analog, analogs with L–D aminoacid substitutions, and analogs that promote the formation of alternative type of β-turn (Sahu et al., 1996, 2000; Morikis et al., 2002).

The structure–activity relations (SAR) determined in our structure-based rational design opened the way for a second round of phage-displayed random peptide library design, which produced a four-fold more active peptide than the parent peptide with sequence Ac-L[CVVQDWG-WHRC]G-NH_2 (Soulika et al., 2003). In this round of design, seven residues were kept fixed while the remaining six residues were randomized. The seven fixed residues were Cys2 and Cys12 of the disulfide bridge, Val3 of the hydrophobic cluster, and Gln5, Asp6, Trp7, and Gly8 of the β-turn. The sequence pattern XCVXQDWGXXXCX was optimized. We also refer to the optimization of the second round of phage-displayed random peptide library as experimental combinatorial design to draw the similarities with the computational combinatorial design (see below) in the selection of the optimization residues. This terminology also distinguishes experimental optimization at the DNA level and computational optimization at the amino acid level.

Compstatin was used as a test case for in silico combinatorial design using a novel two-step computational optimization methodology. This round of design yielded an impressive 16-fold more active analog than the parent peptide with sequence Ac-I[CVYQDWGAHRC]T-NH_2 (Klepeis et al., 2003). The computational methodology has been devised by Klepeis and Floudas (Klepeis et al., 1999, 2002; Klepeis

and Floudas, 2003). The success of the selection of the sequence template pattern XCVXQDWGXXXCX that was used in the experimental combinatorial design and was derived from rational design prompted us to use the same template in the computational combinatorial design. Here again only residues marked with X were allowed to vary. The two steps of the method involve: (i) the selection and ranking of several potential sequences compatible with the sequence template pattern and the NMR-derived structural template. This was accomplished by a mixed-integer linear optimization algorithm based on the use of a backbone potential that was distance-dependent with implicit inclusion of side chain interactions and amino acid specificities (Klepeis et al., 2003). (ii) The calculation of ensemble probabilities for the selected sequences applied on flexible structural templates, which were derived from the NMR structures. This was accomplished by a global optimization algorithm, based on the use of a full-atom force field (Klepeis et al., 2003).

A major finding of the combinatorial design was the identification of pairs or triplets aromatic side chains at positions 4, 7 and 9, which yielded analogs with higher inhibitory activity than the parent peptide. In the case of the most active analog from experimental combinatorial design Trp residues occupied both positions 7 and 9. In the case of computational combinatorial design the pair Tyr4–Trp7 was observed in three analogs and the triplet Tyr4–Trp7–Phe9 was observed in two analogs with higher inhibitory activity than the parent peptide (Klepeis et al., 2003). This observation implicates aromatic rings and possibly their relative orientation in binding and inhibitory activity.

Recently, based on the available structural–functional information, we have synthesized new compstatin analogs, some of which were also expressed in *E. coli* using an intein-mediated expression system (Chong et al., 1998). This approach yielded us an analog that is about 250 times more active than the original compstatin (Katragadda et al., unpublished observations).

Finally, a different round of in silico studies was performed using molecular dynamics simulations, in our effort to understand the dynamic character of compstatin (Mallik et al., 2003). The ensemble of NMR structures, including their averaged minimized structure and the global optimization structure were used (Morikis et al., 1998; Klepeis et al., 1999). The molecular dynamics simulation revealed the presence of five major families of inter-converting conformers at 1 ns of simulation time using implicit solvation model (Mallik et al., 2003). The major conformers were: (i) coil conformation with a Type I β-turn, (ii) β-hairpin with Type II′ β-turn, (iii) β-hairpin with Type I β-turn, (iv) β-hairpin with Type VIII β-turn, and (v) partial α-helix-partial coil. The conformation with largest population (i) was in agreement with the estimated population of the major conformer of compstatin from the original NMR data using averaged NMR observables. It should be noted that 91% of the MD conformers contained some type of a β-turn and 61% contained a Type I β-turn (Mallik et al., 2003).

The molecular dynamics studies demonstrated the significance of the presence of a turn for structural stability of compstatin, while the remaining region can form coil or β-hairpin structures. In the case of the helical structures, one or one-half helix turn can be viewed as equivalent of the β-turn. The molecular dynamics data also provided a measure of the backbone motional amplitudes that were responsible for the inter-conversion among the different conformers. Finally, the molecular dynamics simulations evaluated the free energies of each conformation and the energy barriers that separate the calculated conformers, which is a quantitation of stability (Mallik et al., 2003). These data introduced the concept of a dynamic peptide in our drug design process as opposed to the widely-used, yet overly simplified, static view of peptides. We plan to incorporate this type of analysis in dynamics–activity relations (DAR) studies.

2.3.2. Studies on the C5aR antagonist AcF-[OPdChaWR]

Recently, we have devoted considerable effort to characterizing the interaction of the complement anaphylatoxin C5a with its receptor C5aR in several biological contexts. In this direction, we have chemically synthesized a small potent C5a antagonist developed by Dr. S.M. Taylor's group (AcF-[OPdChaWR]) (Paczkowski et al., 1999; reviewed in Morikis and Lambris, 2002). This cyclic hexapeptide has been successfully used as a selective inhibitor of C5a-mediated functions in animal models of disease and as a means to discern the involvement of C5a in several pathophysiological processes. In C5aR blockade studies using the acetylated species of the antagonist, it was demonstrated that C5aR is critically involved in the development of sepsis (Huber-Lang et al., 2002), in liver regeneration (Strey et al., 2003) and the anti-phospholipid antibody syndrome (Girardi et al., 2003), as well as in the modulation of CD8+ T cell responses during acute influenza infection in mice (Kim et al., submitted for publication).

2.4. Proteomic approaches for studying protein–protein interactions and "profiling" global protein expression

Proteomics is the study of all or a part of the gene transcript population in an organism or a given tissue sample (Pennington et al., 1997). The basic principle is to separate a complex protein/peptide mixture into its components and then analyse 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 been successfully 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 (Anderson and Seilhamer, 1997).

Appreciating the vast capabilities offered by this novel, high throughput technology in global protein expression

profiling, our laboratory has instigated a proteomic analysis of liver regeneration in an effort to identify crucial signaling pathways, and changes in acute phase response and lipid metabolism after partial hepatectomy in mice.

Liver regeneration is a complex physiological process that recruits multiple and redundant molecular pathways in order to ensure effective restoration of the hepatic architecture and function. Identifying hepatocellular targets that are subject to post-translational modifications and could thereby modulate or enhance the regenerative potential of the liver is of great therapeutic benefit for liver transplant recipients and living donors. In this respect, our study has employed a broad range proteomic approach coupled to MS spectroscopy and has identified proteins, belonging to the proteasome and the peroxisome complex, the acute phase response as well as other intracellular signaling proteins, which are noticeably affected during liver regeneration (Strey et al., 2004). Future investigations will address the functional relevance of the identified proteins for the integrity of the regenerative process and their potential use as therapeutic targets.

Furthermore, to expand our understanding of complement-mediated protein–protein interactions, we have 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.

2.5. Thermodynamic studies on protein–protein interactions of the complement system

To study the energetics of complement protein interaction we have applied Isothermal Titration Calorimetry (ITC), a method used to study energetics of the formation of macromolecular complexes in solution that have association constants in the range $\sim 10^3$–$10^9\ M^{-1}$. It measures the electric power that is required to re-equilibrate the temperature of a binding reaction cell with respect to a reference cell as a function of time, upon binding (Cooper and Johnson, 1994). and yields information on the stoichiometry, enthalpy, association constant, and free energy of binding. Using this technique, we have already characterized the interaction of compstatin with C3. Our studies indicated that the binding is 1:1 and occurs through hydrophobic interactions with possible conformational changes in C3 or compstatin. We have also observed some protonation changes occurring at the binding interface (M. Katragadda and J.L. Lambris, unpublished observations). We are now in the process of extending this analysis to the energetics of various protein–protein interactions, with a goal to obtain the energetic parameters of complement activation and regulation pathways.

3. 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 complement 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 complement but have also turned these interactions to their own advantage. Our laboratory in collaboration with that of Drs. Isaacs, Friedman, and Sahu, has actively engaged in a structural and functional analysis of several viral proteins (VCP/SPICE, gC, and KAPOSICA, respectively) that are involved in C′ evasion.

3.1. Studies on the C3b-VCP interaction and vaccinia virus immune evasion strategies

Vaccinia virus complement control protein (VCP) is a 35 kDa secretory protein of vaccinia virus that contains four short consensus repeats (SCRs) (Kotwal and Moss, 1988). 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 complement mediated lysis of sheep erythrocytes, bind to C4b and C3b, and decay the classical and alternative pathway C3 convertases (Kotwal et al., 1990; Mckenzie et al., 1992). Further studies have indicated that VCP acts as a cofactor in the proteolytic inactivation of C4b and C3b by factor I (Sahu et al., 1998).

It has been suggested that all four SCRs are required for VCP's complement-neutralizing activity To elucidate which SCR domains are involved in abolishing complement-enhanced neutralization of vaccinia virus virions and further delineate the mechanisms of complement inactivation by VCP, we have developed monoclonal antibodies that react with VCP (Isaacs et al., 2003). We used the recombinant VCP expressed in Pichia to vaccinate mice for the development of VCP-specific hybridomas. Ten MAbs were isolated and all recognized VCP on Western blots under reducing conditions as well as native-bound VCP in a sandwich enzyme-linked immunosorbent assay. Three of the 10 MAbs (2E5, 3D1, and 3F11) inhibited VCP's abolition of complement-enhanced neutralization of vaccinia virus virions. These MAbs blocked the interaction of VCP with C3b/C4b. The seven remaining MAbs did not alter VCP function in the complement neutralization assay and recognized VCP bound to C3b/C4b. To understand MAb specificity and mode of interaction with VCP, we mapped the MAb binding regions on VCP. The seven nonblocking MAbs all bound to the first SCR of VCP. One of the blocking MAbs recognized SCR2 while the other two recognized either SCR4 or the junction between SCR3 and SCR4, indicating that structural elements involved in the interaction of VCP with C3b/C4b are located within SCR domains 2 and 3 and 4. These anti-VCP MAbs may have important clinical implications serving as potential

therapeutic inhibitors of VCPs complement control activity and may also offer a novel therapeutic platform for managing vaccinia virus vaccine complications that occur from smallpox vaccination. We also hope to use these monoclonals to further characterize the contribution of VCP in viral pathogenesis.

3.2. Studies on the VCP/SPICE interaction with C3b

The smallpox inhibitor of complement enzymes (SPICE) is encoded by variola virus, the causative agent of smallpox, and was found to have complement modulating activity which is similar to that of VCP (Rosengard et al., 2002). Strikingly, despite the fact that it is 100-fold more potent than VCP in inactivating human C3b, SPICE differs from VCP in only 11 amino acid residues. In order to identify the amino acid residues that account for the significant difference in function between the two molecules, chimeric proteins consisting of VCP and SPICE CCP modules were expressed in an *E. coli* expression system. All proteins retained functional activity and were able to inhibit classical pathway complement activation on an immunocomplex ELISA set. Assaying for the ability of these VCP-SPICE chimeras to inhibit alternative pathway-mediated complement activation we were able to show that the amino acids that are associated with the enhanced activity of SPICE are localized on SCR2 (Sfyroera et al., Molecular Immunology, XX Complement Workshop). Furthermore, performing site-directed mutagenesis studies we demonstrated that all SPICE mutants carrying mutations in various amino acid positions in SCR2 had decreased activity over wild type SPICE, thus indicating that a single amino acid difference cannot account for the overall functional advantage of SPICE over VCP (Sfyroera et al., Molecular Immunology, XX Complement Workshop). Further experiments are currently under way and will shed more light onto the molecular aspects and kinetics of the interaction between VCP, SPICE and C3b.

3.3. Studies on the HSV gC and Kaposica interactions with C3

In an effort to define the mechanism by which two other viral homologues of complement regulatory proteins interact with C3b and contribute to immune evasion and pathogenesis, we are in the process of characterizing the complement-neutralizing function of two viral proteins of the herpesvirus family; gC, a glycoprotein encoded by Herpes Simplex Virus type-1 (HSV-1) (Lubinski et al., 1999) and *kaposica*, a complement inhibitor encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) (Mullick et al., 2003). Studies using animal gene knock out models and expressed recombinant proteins have revealed several aspects of the molecular interaction between C3b and gC/kaposica, and will hopefully contribute to developing effective therapeutics for the treatment of these viral diseases.

4. 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 years.

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* (Sunyer et al., 1996; Sunyer et al., 1997). In all other animals studied so 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 (Sunyer et al., 1996). 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, since the specific immune response in fish is relatively weak compared 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.

4.1. In search for complement receptors in lower vertebrates

Complement anaphylatoxins mediate their proinflammatory functions by binding to G-protein coupled transmembrane receptors expressed mainly on blood derived leukocytes. Their function has been thus far characterized only in mammalian systems since no such receptors have been cloned or isolated in lower vertebrates. Our laboratory has been rigorously engaged in identifying such receptors in species other than mammals and recently, evidence was produced suggesting that inflammatory pathways mediated by anaphylatoxic peptides are present both in teleost fish and urochordates, an invertebrate species.

In collaboration with Dr. M.R. Pinto (Stazione Zoologica, Napoli, Italy), we recently identified two C3-like genes in the invertebrate urochordate *Ciona intestinalis* (Marino et al., 2002). To define the functions mediated by these innate immune molecules in urochordates we expressed the C3a moiety of Ciona C3-1 (CiC3-1a) and found that it can stimulate the dose-dependent directional migration of granular amebocytes (hemocytes) in a manner similar to mammalian C3a. The chemotactic activity of this peptide was localized to the C-terminus, and pretreatment of Ciona hemocytes with pertussis toxin abolished the chemotactic response to C3a, suggesting that this effect is mediated by a Gi-protein coupled receptor. This is the first report documenting the

presence of a C3aR like receptor in an invertebrate species (Pinto et al., 2003).

Furthermore, studies performed in our laboratory have demonstrated the presence of a functional C5aR receptor in a teleost species, the rainbow trout. Recombinant trout C5a was able to induce the chemotactic response of trout granulocytes isolated from a head kidney cell population in a dose dependent fashion, and C5aR receptor expression was localized by means of flow cytometric analysis in granulocyte/macrophages cell suspensions of the head kidney as well as in trout peripheral blood leukocytes (Holland and Lambris, 2004).

Taken together, these results strongly indicate that the inflammatory pathways mediated by C3a and C5a are also present in lower vertebrates, further underlying the fact that throughout evolution complement anaphylatoxins and their receptor-mediated pathways have retained a high degree of structural and functional conservation.

5. 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 and developmental processes in various tissues and cannot be clearly placed in an "inflammatory" context (Mastellos and Lambris, 2002; Lee et al., 2004).

Over the past 5 years, our laboratory has been actively engaged in studies to delineate the role of various complement components in three distinct developmental processes: these include limb and lens regeneration in urodeles, liver regeneration in mammals and stem cell differentiation during hematopoietic development.

5.1. The role of complement in liver regeneration

The liver is one of the few organs in mammals that has retained the ability to regenerate, restoring its functional integrity after partial hepatectomy, viral or acute toxic injury (Fausto and Webber, 1994). The hepatic parenchyma reacts to these insults by eliciting a robust proliferative response that causes previously quiescent hepatocytes to re-enter the cell cycle and divide. Several cytokine, hormonal and growth factor-dependent pathways have been implicated in triggering liver regeneration (Cressman et al., 1996; Yamada et al., 1997; Taub et al., 1999).

However, to this date, the potential interaction of such hepatocellular regenerative pathways with components of the innate immune response has not been rigorously addressed.

Our laboratory has previously shown that complement is critical for the normal recovery of the liver after acute toxic challenge (Mastellos et al., 2001). Recently, we went on to establish that complement components are essential priming partners in the early growth response of the liver by showing that complement-mediated pathways are coupled to the cytokine network that drives the regenerative response of hepatocytes (Strey et al., 2003). This study revealed a pivotal role for both anaphylatoxins C3a and C5a in promoting the growth response of regenerating hepatocytes. Furthermore, we demonstrated that blockade of C5aR leads to abrogation of regeneration by affecting the normal induction of cytokines and the early activation of hepatic transcription factors that are essential for the 'priming' of quiescent hepatocytes (Strey et al., 2003).

Further delineating the mechanisms by which complement proteins and receptors interact with other signaling networks in the regenerating liver will provide new insight into the molecular pathways that drive the early growth response of the liver and 'prime' quiescent hepatocytes to re-enter the cell cycle and proliferate.

5.2. Complement and urodele regeneration

The ability to regenerate complex structures and reconstruct entire body parts from damaged tissues is a trait widely encountered among invertebrates (e.g. annelids, hydroids etc.) and in lower vertebrates such as amphibians (Brockes, 1997). In urodele amphibians (axolotls) the process of regeneration is quite prominent in the limb, tail, and in structures of the eye (retinal epithelium and lens) (Tsonis, 1996, 2000). Limb regeneration in urodeles entails the activation of complex developmental pathways that act in concert to promote dedifferentiation, proliferation, and redifferentiation of mesenchymal cells into the specific cell types that comprise the various tissues of the regenerating limb.

The molecular pathway(s) that underlie these developmental stages are largely unknown. In a previous study, our laboratory in collaboration with that of Drs. P. Tsonis (University of Dayton, Ohio) and K. Del Rio-Tsonis (University of Miami, Ohio), has shown that the complement component C3 is expressed in the blastema cells located in the regenerative zone of the amputated limb (Del Rio-Tsonis et al., 1998).

To further study the effect of complement in urodele regeneration and to dissect the specific involvement of the critical components C3 and C5 in limb and lens regeneration, we chose the newt (*Notophthalmus viridescens*) as a model organism that possesses extensive regerative capacity in both these tissues. To study the expression during newt lens and limb regeneration, we isolated newt cDNAs for complement C3 and C5 and generated antibodies against C3a and C5a; these antibodies have been shown to be specific for C3 and C5, respectively, and found to inhibit their activation (Kimura et al., 2003). Expression of both proteins was demonstrated in limb and lens structures during

regeneration by immunostaining using the respective 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 found to cause a significant delay in limb regeneration. In contrast, similar treatment before lentectomy resulted in bigger fiber formation in the lens. To dissect the role of C3 and C5 in regeneration, we are in the process of analyzing the effect of anti-C3a and C5a antibodies in both limb and lens regeneration (Madhavan et al., 2003).

5.3. 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 (Sun et al., 1999) is well appreciated. Very little is known, however, about the distribution of complement components in early hematopoietic progenitor cells, their potential role in hematopoietic development, and the complement-mediated interactions that influence the homing of lymphoid progenitors to various tissues.

We have recently been engaged in profiling the expression of various complement components and receptors in normal human early stem/progenitor cells as well as in lineage-committed hematopoietic cells. In this way, we were able to show that the G-protein-coupled receptors for both C3a and C5a anaphylatoxins are expressed by human clonogeneic $CD34^+$ cells, and that both complement components C3 and C5 are locally synthesized by the bone marrow stroma (Reca et al., 2003).

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 *a*-chemokine stromal-derived factor-1 (SDF-1) and C3a modulates various homing activities of stem cells by increasing their sensitivity to low doses of SDF-1 (Zou et al., 1998; Reca et al., 2003).

These striking observations have laid the groundwork for further investigation of the hypothesis that a functional cross-talk 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.

Corroborating this hypothesis, recent studies have shown that C3a and its receptor C3aR, promote the retention of hematopoietic progenitor/stem cells in the bone marrow during stem cell mobilization in mice (Ratajczak et al., 2004). C3−/− or C3aR−/− mice exhibit increased release of bone marrow progenitors into circulation following GCSF-induced mobilization. Furthermore, mice treated with a specific C3aR antagonist show accelerated mobilization of hematopoitic stem cells (HSC) to the periphery, suggesting that antagonists of the C3aR can potentially serve as mobilizing agents for HSC transplantation (http://www.lambris.com/papers/C3a-patent-application.pdf).

6. Perspectives

In recent years, it is becoming evident that “cutting edge” biomedical research cannot be conducted with the exclusive use of traditional experimental approaches. The enormous amount of raw data accumulating in nucleotide and protein databases has urged the contemporary scientist to adopt a more global and cross-disciplinary approach to “old” scientific questions. Resolving the fine structure and biochemical properties of proteins may still contribute to addressing functions that underlie complex processes, provided that these research components are placed into a wider context of interacting systems and pathways.

In this respect, our laboratory has been engaged in defining the structure–function relationships of several complement proteins and probing the protein–protein interactions that underlie critical functions of complement in diverse biological contexts. Using a combination of biophysical and computational approaches, together with high throughput screening technologies and in vivo animal models, we have been able to verify and also predict the activity of a new generation of complement inhibitors and provide a versatile platform for developing effective complement therapeutics. Furthermore we have generated essential reagents that will help us study C3-mediated effects in mouse models of disease and determine those structural modules that are responsible for these functions (Mastellos et al., 2004). Applying proteomic analysis as a novel tool for probing protein–protein interactions we provide evidence that complement can interact with networks that affect complex developmental processes, such as liver regeneration. In conclusion, all these diverse studies are evaluated under the unifying theme of evolution that provides further insight into the molecular aspects of complement function and underscores the fact that complement—despite its ancient origin—has evolved into a versatile and yet ‘unpredictable’ innate immune system.

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