Review: Assessment of complement activation in clinical immunology laboratories: time for reappraisal?

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Proteins of the complement system have a key role in the mediation of immunologically provoked tissue damaging reactions in human disease. In these, the intensity of the inflammatory process and the nature of the inciting stimulus are reflected in the degree of complement activation and the pathway involved. Despite the potential wealth of information to be gained from direct measurement of pathway specific complement activation, however, current practice still relies on elementary approaches which are of limited usefulness. Numerous new methods, which make use of technology which is accessible to most laboratories, are now available and provide information beyond that supplied by current indirect methods, which often fail to show complement activation. The use of these has meant that it is possible to derive an objective guide to disease activity in several disorders and to enhance our understanding of the pathogenic processes involved. A reappraisal of present approaches to the assessment of complement activation and the adoption of new methods should enable the diagnostic immunology laboratory to provide clinicians with better information on which to base their diagnosis and management.

Introduction

The complement system is one of the principal effectors of the humoral arm of the immune system and is important in defence against microbial infection.¹⁻⁵ Like other components of the immune system, however, complement is a "double-edged sword", and it is its role in the pathogenesis of disease that is of considerable relevance to clinical immunologists. In various disorders in which tissue damage has an immune basis, the role of the complement system as a pathogenetic effector is confirmed by evidence of activation of the cascade.⁶⁻⁸ In this review we set out to illustrate, in the setting of the clinical immunology laboratory, the importance of being able to assess both

the degree and route of complement activation. The current techniques used to detect complement consumption in diagnostic laboratories have been exposed as inadequate in the light of several new approaches. The adoption of new techniques should enable the immunology laboratory to offer clinicians better information regarding the activity and pathogenesis of numerous diseases. This approach is required if the relevance of complement to the diagnosis and management of human disease is to be fully exploited.

The complement cascade

There are three recognised pathways in the complement system: the classical,⁹ alternative,¹⁰ and common or membrane attack pathways¹¹ (figure). Detailed descriptions of the sequential activation of complement may be found elsewhere.¹²⁻¹⁴ The complement system is a true cascade¹⁵ in that it has a continuous, low level of turn-over which is accelerated when activated.¹ The classical pathway is activated by immune complexes and the alternative by bacterial lipo-polysaccharides, both pathways converging at the level of C3 and ultimately leading to the formation of the membrane attack complex (MAC). The principal biological effectors arising from the cascade are the anaphylatoxins (C4a, C3a, and C5a) which induce the release of mast cell mediators and the recruitment of phagocytes, opsonic molecules (C3b) which enhance phagocytosis of micro-organisms, and the MAC (C5b6789) which causes lysis of target cells.

Complement as an effector in disease

The complement system is involved in human disease on two main fronts. Numerous disorders are a direct consequence of deficiency of one or more components of the cascade.^{16,17} In this review, however, we will concentrate on disease which results from inflammation initiated or perpetuated by complement activation.

Figure Diagram of the complement cascade showing activation products which provide unequivocal, pathway specific evidence of complement activation. The classical pathway is activated by immune complexes (IC) with the generation of C4a and C4d from C4, and the complex C1rC1sC1-INH. The alternative pathway is activated by bacterial lipopolysaccharides (LPS) with the production of the complex C3bBbP and fragment Ba from factor B. Both pathways result in the enzymatic cleavage of C3 with the generation of C3a and C3dg and formation of C5a and the membrane attack complex, C5b6789.

There is experimental evidence to show that complement has a central role in the incitement of inflammation and tissue damage in both human disease and animal models.^{18,19} In animal models such as immune complex mediated vasculitis, experimentally induced glomerulonephritis, and Arthus-type reactions, depletion of complement before the provoking stimulus abrogates the development of cellular infiltration and tissue damage.²⁰⁻²² In experimental glomerulonephritis subepithelial deposition of immune complexes seems to initiate a complement dependent mechanism, which mediates the development of proteinuria independently of neutrophils or macrophages.^{23,24} In this model proteinuria correlates with evidence of formation of the MAC and depends on an intact terminal complement pathway.^{25,26}

In several human diseases there is also strong evidence that complement is involved in tissue damage. Systemic lupus erythematosus (SLE), of which glomerulonephritis is a potentially lethal complication, is characterised by the presence of serum

antibodies to double stranded DNA.²⁷ The presence of IgG and DNA, as well as C1q, C4, C3, and evidence of MAC assembly in the glomeruli of patients with lupus nephritis, indicate that complement is activated by immune complexes containing DNA deposited within the kidney and that it has a direct role in renal damage.²⁸⁻³⁰ Assembly of the MAC has also been shown in membranous nephropathy and anti-glomerular basement membrane nephritis.³⁰ Immune complexes and complement factors have been shown in the synovial fluid in rheumatoid arthritis and in the uveal tract in uveitis, both sites of intense inflammation in diseases characterised by the presence of circulating and tissue immune complexes.³¹⁻³³

Measuring complement activation: clinical considerations

It is apparent from the involvement of complement in human disease that information about the activity of the complement system might be of use in a clinical setting. First, the role of complement in inducing tissue damage implies that the degree of activation should reflect the intensity of the inflammatory process. Secondly, because the route of activation is determined by the nature of the stimulus, it becomes possible to know what the immunopathological mechanisms of a disease might be.

A quantitative rather than qualitative assessment of complement activation should give an index of disease activity in those disorders in which complement has a role in the mediation of tissue damage and clinical manifestations. Indeed, several studies have provided evidence of this. Particular attention has been focused on SLE, the prototypic immune complex disease in which disease activity correlates with complement activation when assessed by measuring the metabolic turnover of C3,³⁴⁻³⁷ plasma concentrations of the MAC,^{38,39} the anaphylatoxins^{40,41} and the classical pathway conversion product C4d.⁴² In these studies an increased concentration of conversion products such as the MAC, the anaphylatoxins, and other fragments provides proof of complement activation (see below). In rheumatoid arthritis study of the metabolic turnover of C3 has shown that there is an association between disease activity and complement conversion³⁶; circulating immune complexes and severity of clinical disease have been shown to correlate with C3dg (C3d) and C4d.⁴³⁻⁴⁵ Systemic sclerosis is a connective tissue disease in which complement activation has also been shown to occur by measuring the conversion products C4d, Ba, and C3dg. Moreover, significantly higher concentrations of C3dg and Ba are found in the subgroup of patients with the more severe form of the disease.^{46,47} Given the different mechanisms of activation of the two early complement pathways, useful information may be gained about the patho-

genesis of an immune mediated disease by measuring activation and determining which pathway is predominantly involved. For example, activation occurring largely through the classical pathway suggests the involvement of immune complexes, while activation predominantly via the alternative route is characteristically found in disorders such as Gram negative sepsis.⁴⁸⁻⁵⁰

Current approaches to the measurement of complement activation

The two main approaches to the assessment of complement activation in current use are the determination of complement haemolytic function and the measurement of intact components. In both cases activation is shown indirectly by detecting a reduction in the activity or concentration of components of the cascade. Neither of these approaches, however, can provide accurate information about the extent of complement activation.

The ability of the complement cascade to mediate the lysis of sheep red blood cells coated with haemolysin has been exploited in haemolytic complement assays, the most widely used of which is the Complement Haemolysis 50 (CH50) assay.^{51,52} Reduced haemolytic activity in serum suggests complement consumption, but could also result from reduced synthesis of any factor in the C1-C9 sequence. Although such assays have the advantage of assessing the integrity of the whole of the classical and membrane attack pathways, a 50% reduction in a component is required to compromise the haemolytic activity of serum.³ These techniques are also cumbersome and difficult to standardise⁵³ and are therefore better used in screening for complement component deficiency rather than formally measuring complement consumption.

Information, albeit of a limited and indirect nature, can be obtained by measuring intact complement components. This is the most widely used approach for assessing activation in routine diagnostic practice, usually entailing the measurement of C3 while other components, such as C2 and C5-C9, are only assayed in the investigation of complement deficiency.⁵³⁻⁵⁵ Specific antisera are commercially available for detecting most of the intact components and these molecules have relatively high serum concentrations. Techniques such as radial immunodiffusion and rocket immunoelectrophoresis can be used for their measurement and are in routine use in clinical immunology laboratories.^{54,55} Nephelometry makes use of automated instrumentation and is more amenable to the analysis of large batches of samples. The limitations of this approach in the assessment of complement activation are becoming apparent.^{17,56,57} First,

there is a wide normal range in the concentration of these components (in our laboratory, for example C3 0.6-1.2 g/l and C4, 0.2-0.6 g/l). Thus unless a normal value has been obtained from a particular patient during a period of health, concentrations of complement factors may be reduced by 50%, representing extensive activation, but may still fall within the normal range. Complement activation of any lesser magnitude, while of considerable clinical importance, would go undetected by this method. Secondly, proteins of the complement system act as acute phase reactants, resulting in increased synthesis during episodes of inflammatory or infective diseases.⁵⁸ Such an increase could compensate for extensive catabolism, which occurs when there is a simultaneous activation of the cascade, thus masking complement consumption. As a result, complement concentrations may be normal or increased despite concomitant activation. Finally, concentrations of complement factors may be reduced due to an inherited deficiency or as a result of decreased synthesis. Several diseases are associated with inherited deficiencies of complement components due to defective alleles at loci coding for the complement proteins.^{16,17} In SLE, insulin dependent diabetes, and autoimmune chronic active hepatitis low concentrations of C4 are genetically determined⁵⁹⁻⁶¹ and cannot therefore provide accurate information about complement activation. Moreover, decreased synthesis of C3 has been reported in patients with SLE⁶² and decreased synthesis of C3 and C4 in newly diagnosed patients with insulin dependent diabetes.⁶³

Assays which quantitate total haemolytic complement and the concentration of intact components measure static conditions of the cascade. If an analogy is made between complement activation and the generation of hydroelectric power, this approach is akin to measuring the water level in the reservoir above the dam. The water level depends on the influx from streams and the outflow through the dam, and its measurement does not provide any evidence as to how much electricity is being produced by the flow of water through the turbines. On purely theoretical grounds, therefore, the static measurement of intact complement components is an inadequate way of assessing a dynamic system. A direct approach which assesses the products of complement conversion, on the other hand, should provide unequivocal evidence that activation is occurring: this is analogous to measuring the electrical power generated by the dam.

The inadequacy of measuring a reduction in haemolytic function and intact components as a marker of activation is illustrated by several studies in which CH50, C4, factor B and C3 have been measured in patients with SLE,^{42,64-66} rheumatoid arthritis,^{44,45,67,68} and systemic sclerosis,^{46,47} including those with severely

active forms of their disease. In none of these studies was there a reduction in the concentrations of the parent molecules, however, even though unequivocal evidence of activation was obtained using other, direct approaches.

Newer approaches to the measurement of complement activation

To summarise thus far, the clinical immunology laboratory should reassess its approach to the assessment of complement activation if it is to contribute usefully to the management of a range of diseases. This requires the adoption of assays which incorporate sensitive and quantitative techniques into a direct approach to detecting complement activation, thus providing a measure of the extent of activation. Such methods must also be applicable to individual complement pathways to give evidence of route specific complement conversion, and be sufficiently practicable to receive widespread application.

The detection of the appearance of products of complement conversion is the logical approach to assessing activation in a dynamic system such as the complement cascade. Activation results in the generation of single fragments as cleavage products and also of functional complexes composed of several factors. Complement activation can thus be assessed by showing the formation of such moieties which afford several advantages in the assessment of complement activation. First, they are only produced as a consequence of activation of the cascade. Secondly, they are produced on an equimolar basis as the intact molecules are cleaved. Thirdly, they are each distinct products of their pathway of origin.

Complement fragments generated by cleavage of larger molecules retain some of the antigenic determinants expressed on their precursors. The cleavage products of C3, for example, all have epitopes in common with intact C3, so that each will be detected using an anti-C3 antiserum. For this reason, fragments must be separated from their respective parent molecules before being measured, and, unless a specific antiserum is available, from other related fragments. Complement fragments can be separated from related molecules on the basis of different properties such as size, charge, and their ability to bind to specific antisera^{69,70} or lectins.^{71,72} Historically, assays have been performed using techniques which rely on the electrophoretic separation of complement fragments in gel, followed by a revealing step using antiserum to the parent molecule, usually C3. These techniques have been reviewed elsewhere⁷³ and they have the disadvantage of being relatively insensitive. They supply largely qualitative or semiquantitative information based on the presence or absence of lines

of precipitation representing split products, as well as being slow and cumbersome to perform. In practical terms, therefore, it is now more appropriate to determine cleavage products such as the anaphylatoxins, C4a, C3a, and C5a, and the fragments C4d, Ba, and C3dg as these are easily separated from larger related molecules by virtue of their low molecular weight without the requirement for electrophoresis. In addition, at least in the case of C4d, Ba, and C3dg, neo-antigens arise during cleavage and can be exploited for detection purposes. Alternatively, similar information can be obtained by measuring functional moieties including the complex of C1rC1s inactivated by C1-inhibitor (C1rC1sC1-INH), the alternative pathway C3 convertase (C3bBbP), and the MAC (C5b6789).

ANAPHYLATOXINS

Assays have been developed to measure C4a, C3a, and C5a, the molecules directly responsible for some of the major biological effects of complement. Radioimmunoassays measuring the concentrations of C4a, C3a, and C5a are performed by incubating the fragment in plasma with a known concentration of the same ligand labelled with ¹²⁵I.⁷⁴⁻⁷⁷ Addition of specific antibody results in competition for binding sites and anaphylatoxin in the plasma competes with and displaces the radiolabel. Free and antibody-bound ligand are separated using affinity chromatography or ammonium sulphate precipitation, and the radioactivity associated with the antibody is measured. The technique, which has a lower limit of sensitivity of 10 ng/ml,⁷⁷ has been used to show the presence of increased concentrations of anaphylatoxins in the urine of patients with SLE and in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome.⁷⁷ The assays have been able to provide useful information regarding the pathogenesis of various immune disorders, such as SLE^{40,41} cardiopulmonary bypass syndrome,⁷⁴ and pancreatitis.⁷⁸ A disadvantage inherent in these assays is the use of radioactive compounds. Despite the sensitivity of the radioimmunoassays, they are also often unable to detect the biological fragments in normal plasma because the anaphylatoxins have short half-lives⁷⁹ and bind to high affinity receptors on phagocytes and mast cells.⁸⁰

C4d, Ba, AND C3dg

Problems encountered in assessing complement activation by the measurement of the anaphylatoxins can be circumvented by an alternative approach: the measurement of split products which, though relatively inert as far as biological activity is concerned, are produced at the same time as the anaphylatoxins and are more easily detected. Fragments such as C3dg have long half-lives and remain in the circulation

at relatively high concentrations once generated.⁷⁹ Isolation by polyethylene glycol precipitation (PEG) (6000 kilodaltons) enabled Perrin *et al* to measure concentrations of the fragments C4d, Ba, and C3dg in patients with rheumatoid arthritis,⁸¹ SLE, and glomerulonephritis⁸² using radial immunodiffusion. Subsequently, sensitivity has been considerably enhanced by the use of nephelometry^{50 83 84} and ELISA^{85 86} which are sensitive, quantitative, and suited to the analysis of large batches of samples.

Assays have been developed based on PEG separation of C4d, Ba, and C3dg with subsequent quantitation using commercially available antisera and an automated bench nephelometer.^{50 83 84} Evaluation of these split products allows activation to be measured through the classical, alternative, and common pathways.

By determining the concentration of the parent molecules in a matched serum sample, the ratio of fragment to parent molecule can also be calculated. This ratio (C4d:C4, Ba:factor B, C3dg:C3) is particularly valuable because it provides an index of complement activation that is independent of the initial concentration of the intact component. The ratio has been shown to correlate most closely with the fractional catabolic rate of the intact component, as calculated in *in vivo* studies of complement turnover in health and disease.^{37 87}

The use of PEG precipitation, followed by nephelometric analysis of the supernate, allows large batches of samples to be analysed, which favours the use of this technique in the routine laboratory. The techniques are also sensitive, which is advantageous in two respects. First, the low concentrations of complement fragments C4d, Ba, and C3dg found in normal subjects are detectable so that pathological complement activation is clearly identified. Secondly, differences in complement activation between groups of patients divided according to disease activity can be determined. These benefits are illustrated by several studies performed on patients with SLE,⁴² rheumatoid arthritis, and systemic sclerosis,^{46 47} as described above. In particular, concentrations of C4d increase significantly step-wise in patients with SLE of increasing disease activity who are assessed clinically, and, in systemic sclerosis, patients with the more severe diffuse cutaneous skin disease have higher concentrations of C3dg than those with localised disease.

FUNCTIONAL COMPLEXES

The measurement of functional complexes as an approach is both sensitive and elegant when applied in an ELISA, using two antisera directed at different components of the same complex.^{88 89} The use of a sandwich ELISA technique to detect two "sides" of a functional complement complex has enabled assays

investigating the classical, alternative, and common pathways of activation to be developed.^{54 55} In the classical pathway C1 activity is terminated by the binding of C1-INH forming the complex C1rC1sC1-INH which can then be detected using anti-C1s and enzyme labelled anti-C1-INH.⁹⁰ Alternative pathway activation is investigated using anti-factor P and enzyme labelled anti-C3 to identify C3bBbP complexes⁸⁸ which is the alternative pathway convertase stabilised by factor P. In a similar way an assay has been developed measuring the MAC formed when the common pathway is activated.⁶⁶ In this assay wells of microtitre plates were coated with anti-human C9 and the "sandwich" completed using anti-human C5, thus detecting only preformed terminal component complexes. Using this technique, Gawryl *et al* were able to measure concentrations of C5b6789 as low as 0.3 µg/ml, and showed that concentrations were correlated with disease activity in patients with SLR.³⁹ The technique was not sufficiently sensitive to detect complexes in normal controls, however. Modifications by Hugo, Krämer, and Bhakdi⁹¹ have permitted the detection of terminal complement complexes in only two thirds of normal controls.

Future approaches: the detection of neo-antigens

During activation of the complement system, changes in the antigenicity of intact complement components and fragments occur as a consequence of allosteric modifications and cleavage. Some epitopes, such as those on C1q and C3dg, may appear during activation,⁹²⁻⁹⁴ while others may disappear, as is the case for C1r.⁹⁵ Neo-antigens can be used to show complement conversion,⁵⁵ and it is likely that the development of monoclonal antibodies directed against neo-antigens will provide powerful tools for the investigation of complement activation in the future. Assays have been developed to measure C3dg⁹⁴ and terminal complement complex neo-antigens.^{38 89 96-98} A separation step is not required for neo-determinants to be detected and quantitated with accuracy, and an ELISA based on a monoclonal antibody directed to epitopes present on iC3b, C3dg, and C3dg⁹⁹ has thus been used to detect complement activation in high risk samples from patients infected with HIV.¹⁰⁰ Monoclonal antibodies directed against neo-antigens present on C4d and Ba have recently been raised and offer the possibility of exploiting this approach to quantitate complement activation through the specific complement pathways.^{101 102}

Conclusions

By selectively exploiting the assays discussed above, routine immunology laboratories should be able to

offer clinicians better information about the state of complement activation in their patients. The assays provide direct, unequivocal evidence of complement activation and also indicate the degree and route through which it is proceeding. Furthermore, they require technology to which most diagnostic laboratories have access. Assays could also be particularly relevant to those diseases in which complement activation has a role, potentially providing an index of disease activity as well as elucidating aspects of the pathogenesis of immune mediated disease. The conventional approaches which are commonly used to indicate complement consumption are inadequate. A new approach is required if complement, a phylogenetic elder statesman of the immune system, is to retain its relevance during the next decades of technological advances in immunology.

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