Complement: Measurement

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Introduction

The complement system comprises a group of serum proteins and cell membrane receptors that function primarily to fight infection. These components interact in three activation pathways and one final pathway. The central results of activation of these pathways are to deposit the opsonin C3b on bacteria to promote phagocytosis, to lyse bacteria by the assembly of the terminal membrane attack complex and to promote inflammation. Clinically, measurement of pathway activity and individual component levels is, therefore, of value in cases of immunodeficiency and inflammatory conditions that involve complement activation, the prime example of the latter being systemic lupus erythematosus (SLE).

The measurement of complement protein levels is readily achieved using standard immunochemical methods. Nephelometry and turbidimetry can be used to assay levels of serum complement components that are present in relatively high concentrations (e.g. C3, C4, C1-inhibitor (C1-inh)). Enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) are used to measure components present in relatively low concentrations, but serum/plasma concentrations of all components have been successfully measured by ELISA. It is also possible to determine the functional activity of the majority of the complement components. To fully appreciate the conditions required for the functional assays, it is important to understand the process that is occurring. Accordingly a summary of the complement pathways, stressing certain conditions necessary for activation, is given in the following section, with more detailed descriptions to be found in the individual assay sections. Full methodologies for the assays described in this chapter are given in the literature listed for further reading.

The classical pathway

Immune complexes that contain immunoglobulin G (IgG) or IgM can activate the classical pathway by the binding of the first component of complement (C1) via the C1q subcomponent. Once bound, the subcomponent C1r is activated and this, in turn, activates C1s. The integrity of the C1q–C1r–C1s complex is calcium-dependent. Activated C1s is a serine protease that activates C4 by limited proteolysis. Cleaved C4 (C4b) will bind to the activating complex. C2 binds to C4b when magnesium ions are present and C1s will then activate C2 to form the C4b2a complex (classical pathway C3 convertase) that activates C3 by limited proteolysis. Activated C3 (C3b) will bind covalently to certain surfaces (see below) and act as a high-a nity ligand for C3b receptors on phagocytes.

In addition to the inherent instability of C4b2a, spontaneous activation of the classical pathway is controlled in the initial stages by the serine protease inhibitor

The alternative pathway

The alternative pathway of complement activation requires C3, factor B, factor D and properdin. Activation of this pathway involves a constant, low level of spontaneous hydrolysis of a small proportion of circulating C3, which permits binding of factor B in the presence of magnesium ions. Factor D cleaves the bound factor B to form C3Bb and the latter can activate further C3 to form C3a and C3b. The C3b generated in this way can bind to surfaces via a thioester and, once attached, bind more factor B. The thioester bond preferentially reacts with hydroxyl groups to form ester bonds. Surfaces of alternative pathway-activating particles bind C3b in a form that is resistant to the regulatory activities of factors H and I. Sialic acid is one known surface constituent which render surfaces resistant to alternative pathway activation. The membrane-associated regulatory molecules decay-accelerating factor (DAF), CR1, and membrane cofactor protein (MCP) also mediate inactivation of C3b on host cells. Factor B bound to C3b on activating surfaces is cleaved by factor D to give C3bBb. Properdin stabilizes this complex and C3bBbP can cleave further C3 molecules, thus amplifying the process. Additional C3b can bind to the original C3b to alter the specificity of the enzyme to that of a C5 convertase (see below).

The membrane attack complex (MAC)

C5 can be activated by either the classical or alternative pathway by C5 convertases (C4bC3b2a and $(C3b)_nBbP$ respectively). Activated C5 binds to C6 and the remaining components, C7, C8 and C9, bind in turn. At least six molecules of C9 will bind to this complex and form a pore in the membrane of a cell on which it is present. This can lead to lysis of the cell, a property that is used in haemolytic assays. Two membrane-bound regulatory proteins, homologous restriction factor (HRF) and CD59, inhibit self-inflicted cell lysis.

Measurement of Total Complement Activity

The CH_{50} and APH_{50} assays are used to determine whether or not a patient is genetically deficient in a complement component. (CH_{50} and APH_{50} refer to the reciprocal dilution of a serum required to produce 50% haemolysis of a standard preparation of antibody sensitized sheep red blood cells (classical pathway, CH_{50}) or rabbit erythrocytes (alternative pathway, APH_{50}), respectively.) Both the deficiency of a component or the presence of a nonfunctional component will be detected.

Specimen preparation and storage

As some components are extremely labile, incorrect storage of samples for complement assay can result in decreased levels. For assays of individual components in serum, blood samples should arrive in the laboratory as soon as possible after venepuncture. Blood should be allowed to clot at room temperature for 30 minutes, then the sample placed on ice for 1 hour for clot retraction to occur. Separation should occur at 2–4°C. Samples should be aliquotted and stored at −70°C as quickly as practicable and adequate aliquots stored from each sample to prevent thawing and refreezing for each assay. For use, samples should be thawed at 37°C then immediately placed on ice. Blood collected into EDTA (ethylenediaminetetraacetic acid) (which prevents further complement activation by chelating calcium and magnesium and, hence, such samples are suitable for activation product assays) should be kept on ice for as short a time as possible before being spun to produce platelet poor plasma which is stored as for serum. Samples from tissue culture may benefit from the addition of proteinase inhibitors such as phenylmethylsulfonyl fluoride (PMSF).

Haemolytic complement assays

If the MAC is assembled on the cell membrane of red blood cells and polymeric C9 inserted, lysis will occur. This phenomenon is used to assess the functional integrity of the whole of the classical or alternative pathways with the terminal pathway. The simplest of these assays is the CH_{50}. This assay depends on a sample containing all the classical and terminal complement components and depends on the components being functionally active. The assay is quantitative, with the result expressed as the reciprocal of the serum dilution required to produce lysis of 50% of defined numbers red cells under standard conditions. The pathway is initiated by IgM on the surface of sheep red blood cells (EA, antibody sensitized sheep erythrocytes) and the assay is performed in the presence of calcium and magnesium ions (required for classical pathway activation).

Sheep red cells are ine cient at activating the alternative pathway, so rabbit erythrocytes are used in APH_{50} assays. EGTA (ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid) chelates calcium, but not magnesium ions, and will prevent any concomitant activation of the classical pathway while permitting activation of the alternative pathway.

These assays need to be controlled with positive control samples known to contain all the components, e.g. fresh normal human serum (NHS), and by bu er alone (negative control) which controls spontaneous lysis of the erythrocytes. The amount of haemolysis (measured by the optical density of the cell supernatant) can be compared with a known NHS in a one-tube method. Alternatively, using the

von Krogh equation which describes the curve obtained by plotting the percentage lysis against the sample dilution, the sample dilution required to obtain 50% haemolysis is calculated and, after taking the initial sample dilution into account, this value is translated into CH_{50} units mL^{-1} (Figure 1). Controls and standard conditions are essential, as the CH_{50} unit obtained depends on the amount and nature of the antibody used to sensitize the cells, the erythrocyte concentration and fragility, the ionic strength, divalent cation concentration and pH of the bu er, reaction time and temperature.

The above assays can also be performed using single test tubes or agarose gels, either method being suitable for large-scale clinical screening. An automated method for screening classical pathway activity using sensitized liposomes is also available.

Immunochemical assays

ELISA plates coated with a classical pathway activator (e.g. IgM, IgG-containing complexes) or an alternative pathway activator (lipopolysaccharide) can be used to assess the respective pathways by looking for the appearance of neo-epitopes of C9 or properdin upon the addition of serum.

Quantitation of Individual Components

When a serum is shown to be deficient in classical, alternative or terminal pathway activity, then the component that is missing or nonfunctional is defined.

Immunochemical assays

The assay system of choice depends on the number of assays being performed, the level of sensitivity required, the level of the analyte and the quality of the antibody available. For instance, in patient samples, nephelometry is used in routine clinical immunology laboratories for measuring C3, C4, C1-inh and C1q. Although radial immunodi usion (RID) (available commercially) can be used, ELISA is a robust and sensitive technique, especially with regard to quantitation of serum/plasma levels of other complement components. For cell culture studies, however, levels may be as low as 1 ng mL^{-1} and ELISA or RIA may be the only practical method available. The bu ers used in these types of assay are those that are optimal for antibody binding and phosphate-bu ered saline (PBS) is usually used. For all of these assays, the choice of antibody is critical. Polyclonal antibodies are generally used, and these may recognize breakdown products of the component under investigation and the resulting value obtained may not reflect the level of functional protein present.

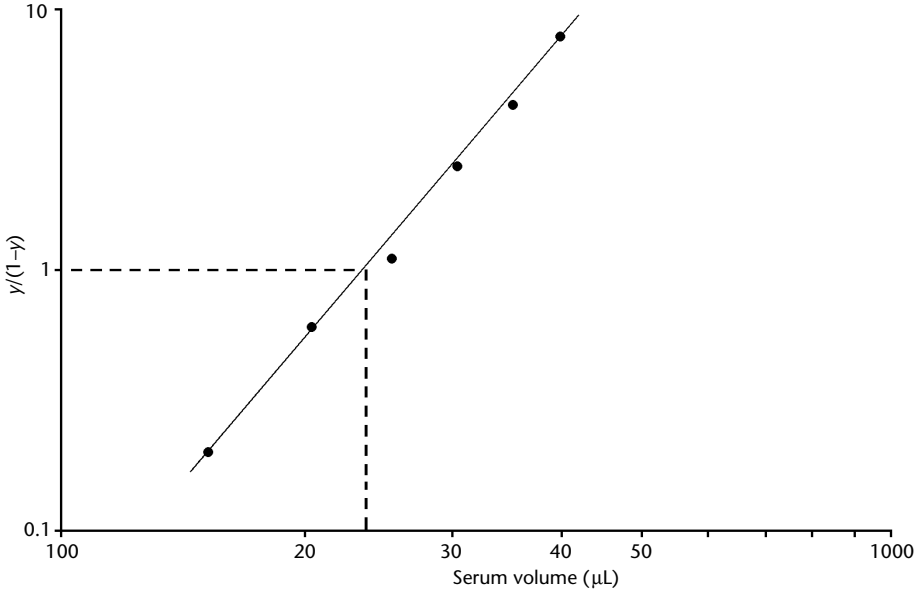

Figure 1 Log–log plot of $y/1 - y$ against volume of diluted serum in a CH_{50} assay. At the point of 50% haemolysis, $y/(1 - y) = 1$ and the volume of diluted serum giving this is shown by the vertical line. Redrawn from Whaley K (ed.) (1985) *Methods in Complement for Clinical Immunologists*, p. 102. Edinburgh: Churchill Livingstone.

Other techniques

Gel rocket techniques, although time consuming and not especially sensitive, are relatively simple. Abnormal forms of complement components can be detected by western blots but Ouchterlony (a protein-detection method) can occasionally be as useful. For example, NHS and C8-deficient serum against anti-C8 may reveal lines of partial identity, suggesting C8 β chain deficiency.

Detection of complement activation products

Measuring products of complement activation by using antibodies that distinguish activation products from the native molecule can assess increased complement turnover. Before a good range of discriminatory antibodies was available, cleavage products and native molecules could be distinguished by di erent electrophoretic mobility. Commercial kits are available, but inhouse assays for activation products are easily established, provided appropriate antibodies are used. ELISAs and RIAs for C3a, C4a and C5a can be established, provided standards can be obtained, but the clinical usefulness of these assays has not been fully evaluated. Precautions to prevent generation of anaphylatoxins *i i* are essential. Crossed immunoelectrophoresis or preincubation of serum samples with anti-C3c/polyethylene glycol (PEG) allows the quantitation of C3d after the C3c has been removed. Of more help to clinicians are ELISA assays for stable, macromolecular complexes formed during complement activation. The quantitation of C1s–C1-inh (classical pathway), C3bBbP (alternative pathway), or C5b–C9 (terminal pathway) complexes indicates the degree of activation of each pathway. The latter assays use an antibody against one component (e.g. C1s) to bind the complex to an ELISA plate, and an antibody against another component of the complex (e.g. C1-inh) to detect the intact complex.

Detection of complement receptors

Monoclonal antibodies to complement receptors are available commercially and can be used in flow cytometry to detect the presence of receptors on cell suspensions. Care must be taken in obtaining the specimens that activation of the cells and consequent upregulation of the receptor does not occur. Antibodies are also suitable for immunohistochemistry of tissue sections or cytospin preparation if cell morphology is required.

Haemolytic assays for individual components using complement-deficient sera

The simplest haemolytic assays for assessing both the presence and functional activity of individual components use EA and a serum deficient in the chosen component. Adding all other components in excess together with serial dilutions of a test sample will give lysis of EA only if the tested-for component is present and functional. As other components are present in excess, lysis is proportional to the amount of test component. Sera that are deficient in individual components can be obtained commercially, but can also be prepared in the laboratory, if such assays are performed regularly. These assays are not as sensitive as those using preformed defined haemolytic intermediates and are not suitable for regulatory components (C1-inh, factors H and I, properdin).

Haemolytic assays for individual components using haemolytic intermediates

Complement components can be added to erythrocytes in a step-wise fashion. If the components in either the classical or alternative pathway are added up to the level of the component under test, the test component will bind if subsequently added. Addition of the remaining components in the pathway will result in lysis if the test component is present and functional. As for the above assays, by ensuring that all other components are in excess, the degree of lysis is proportional to the amount of test component. Most are functionally pure components commercially available, but all can be prepared in the laboratory if there is su cient requirement. EAC1, EAC4 and EAC14 are IgM-sensitized sheep erythrocytes coated with C1, C4 and C1 with C4, respectively. Using a low ionic strength bu er that contains calcium and magnesium (usually veronal (barbitone) bu er), EAC1 are prepared by adding C1 to EA. (Note that barbitone bu er is extremely poisonous.) After washing in bu er that does not contain calcium, the addition of human serum in the presence of EDTA will result in the binding of C4 and C2. Subsequent incubation at 37°C will cause decay of C1 and C2 from the cells, leaving EAC4. Adding back C1 in the presence of calcium ions generates EAC14 cells. EAC1 and EAC14 must be kept on ice and used within a few hours while EAC4 can be kept at 2–4°C for up to 2 weeks.

C1 activity is assayed by adding dilutions of the test sample to EAC4 cells, adding guinea-pig C2 and, finally, adding the remaining components, usually in the form of rat serum containing EDTA (C^{rat}-EDTA). To calculate the concentration of e ective molecules of the component under test, the Z value ($-\ln(1 - \%\ \text{lysis})$) is plotted against the dilution of the component under test (Figure 2). As a single complement molecule (C1–C9) can e ectively lyse an individual cell, a straight line is achieved. One unit of complement is taken as that which gives 63% lysis ($Z = 1$) in an assay for that component. If a straight-line plot is not obtained then the concentration of one or more components not under investigation is limiting.

Similarly, C4 is assayed using EAC1, dilutions of the test sample, guinea-pig C2 and C^{rat}-EDTA as for C1. C2

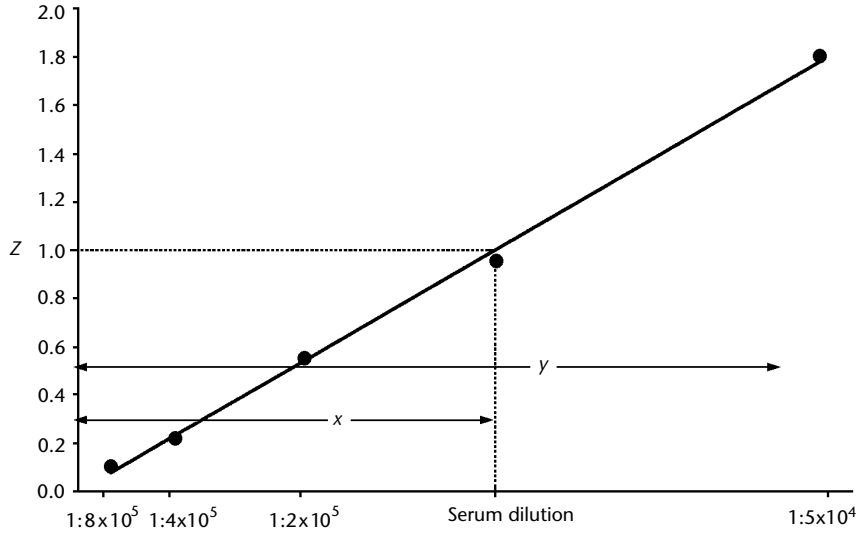

Figure 2 The number of functional molecules per cell (*Z*) is plotted against the serum dilution in a typical haemolytic titration of an individual complement component. The concentration of the component in this instance is given by $y/x \times 50\,000$. Redrawn from Whaley K (ed.) (1985) *Methods in Complement for Clinical Immunologists*, p. 107. Edinburgh: Churchill Livingstone.

activity is measured using EAC14 but in this case the time for maximum C4b2a formation (T_{max}) for the cells must be known. The T_{max} depends upon the amount of C4b present on the cells (Figure 3). As the C4b2a complex is more unstable than most, $EAC14^{oxy}$ 2 are prepared to provide increased haemolytic activity of C2, required for the measurement of C3 activity.

For alternative pathway activity, C2 and C3 are added to EAC14 to form EAC1423 with subsequent removal of C1 and C2 by incubation in EDTA bu er. A stable C3 convertase is formed on the resultant EAC43b if factor B, factor D and properdin are added. Haemolysis is achieved by adding C^{rat}-EDTA. Samples can be assayed for the activity of factor B or properdin by adding dilutions of the test sample and omitting the respective pure component. Factor D activity in serum cannot be measured by this method and, therefore, only pure preparations of factor D are suitable.

Terminal components are assayed by adding C3 and the terminal components up to the one under test to $EAC14^{oxy}$ 2. EAC1–8, used for measuring C9 activity, will undergo spontaneous lysis so must be used as soon as they are prepared.

Functional assays of complement control proteins

The ability of plasma C1-inh to inhibit C1s or a C1s analogue is used in commercial kits or inhouse assays in colorimetric reactions. Some of these assays are easily adapted to look for the presence of anti-C1-inh antibodies. Very rarely, dysfunctional C1-inh variants may inhibit C1s in some of these assays.

In a more technically demanding assay, C1-inh activity can be measured by testing the ability of the test sample to inhibit a given level of exogenous C1 added to sensitized EA. If serum is being tested then C1 first has to be removed, e.g. by precipitating C1 out using phosphate bu er. C1-inh can be allowed to bind to C1 either in the fluid phase before adding to EAC4 with C2 or on EAC14, prior to the addition of C2. For haemolytic inhibitor component assays generally, a 'solo' reaction that has no inhibitor is run in parallel for direct comparison.

Factor H levels can be assayed by measuring the ability of serum to accelerate the decay of the C3 convertase on erythrocytes. EAC4b3bBbP are prepared by adding properdin, factor B and factor D to EAC4b3b. The amount of haemolytic activity remaining after incubating the sensitized cells with the test sample is compared to a solo tube. Similarly, factor I activity can be assayed by incubating the test sample with factor H added to EAC43 to inactivate the C3. Factors B and D are then added in order to complete the alternative pathway and any residual haemolytic activity indicates a deficiency of the inhibitor.

Clinical Relevance

Immunodeficiency

The authors recommend that any individual experiencing serious and/or recurrent sepsis, for which no other cause is identified, should be tested using a CH_{50}/APH_{50}. This is especially important in cases of meningococcal meningitis, which is the commonest presentation of terminal pathway component deficiencies and properdin deficiency (Ross and Denson, 1984). An absence of CH_{50} activity, but normal APH_{50} activity, indicates a deficiency of C1q, C1r, C1s, C4 or C2 (the latter being the commonest complement component deficiency). In contrast, a lack of APH_{50} activity but normal CH_{50} suggests a deficiency of properdin or, less commonly, factor D. No activity in either assay indicates a deficiency in C3 or one of the terminal components. In practice, C9 deficiency is associated with some lysis of the test cells. Low values above zero suggest that the level of one or more complement components is either decreased due to consumption in a disease state or may indicate a heterozygous deficiency state. As many complement components are acute phase proteins, the detection of heterozygous deficiency states is best achieved by genotyping, rather than by measuring levels of the protein.

Deficiencies in the classical pathway are associated with sepsis and/or immune complex disease (Pickering and Walport, 2000). C2 deficiency is more often associated with increased infection and less often with immune complex disease compared to deficiencies of C1 and C4. Asymptomatic deficient individuals are often defined in family studies, the exception being C3 deficiency which is invariably associated with recurrent sepsis. Factor H deficiency results in secondary C3 deficiency and an increase in infections, but is primarily associated with autoimmune haemolysis, often as part of the haemolytic–uraemic syndrome.

MBL deficiency is also associated with recurrent infections in children and some adults but only in some cases. The inconsistency in the clinical e ect on individuals seen in complement deficiency suggests other factors are important in defining the phenotype seen but, as yet, no clear associations have been found.

Deficiencies of CR3 (CD11b/CD18) and CR4 (CD11a/CD18) are associated with severe recurrent bacterial infection and poor wound healing. Expression on peripheral blood neutrophils and monocytes can be routinely

deficiency). More commonly seen is the association between C4-null alleles and connective tissue disease such as SLE and systemic sclerosis. In these conditions, CR1 expression on red blood cells is also seen, particularly during episodes of disease exacerbation. C3 and especially C4 are consumed during active disease and serial C3 and C4 levels are markers of disease activity. C4 may, however, be low due to genetic reasons as mentioned above and both C3 and C4 are acute phase proteins so that increased production may mask consumption. It has now been established that measuring activation products such as C4a or C3 degradation products is a more sensitive way of monitoring disease activity. Assays for C3dg are generally robust, providing specimens are handled correctly, and there is some evidence that increases in levels may predict clinical relapse in SLE.

C1-inh deficiency

Patients presenting with nonpainful, nonitchy angioedema should be investigated for C1-inh deficiency (Cicardi *e al.*, 1998). Low C4 and C2 levels are seen during acute attacks and usually between attacks. C1-inh levels are low in 85% of cases of hereditary C1-inh deficiency (HAE) but the remaining patients have normal levels of a nonfunctional protein and functional assays are required for the correct diagnosis. Acquired C1-inh deficiency (AAE) is associated with either B cell neoplasia, connective tissue disease or various carcinomas, or may arise as an autoimmune condition *e e*. Previously divided into two subtypes, it now appears that AAE is always associated with autoantibodies to C1-inh. The antibodies a ect C1-inh function directly and result in an inactive form of C1-inh of a lower molecular weight than normal. C1q levels are usually decreased more in AAE than in HAE. The antibody can be detected by ELISA, immobilizing C1-inh on the plate, incubating with patient serum and detecting bound antibody by enzyme-labelled anti-IgG, IgA or IgM.

C3 nephritic factor

Patients with membranoproliferative glomerulonephritis, especially type II and associated with partial lipodystrophy, often show a secondary C3 deficiency. This is usually because of an autoantibody against C3bBb. The antibody can be detected by its ability to activate fluid phase C3 or the stabilization of sheep erythrocyte-bound C43bBb. An ELISA using stabilized C3bBb as a substrate has also been described.

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