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Complement analysis in the era of targeted therapeutics

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ABSTRACT

Complement immunobiology, and with it complement analysis, has undergone a renaissance in the past decade. Classically, complement analysis was limited number of testing C3, C4 in a routine laboratory with the possible addition of CH50 with all other analysis being performed at only few highly esoteric laboratories. This diagnostics expanding beyond specialized laboratories is the result of the growing recognition of the role played by complement dysfunction in many more diseases and disorders and the concomitant increase in interest in complement targeting therapeutics. In response, laboratories specializing in complement analysis have joined with the International Complement Society and the IUIS to coordinate efforts to standardize and improve complement testing, ongoing efforts that have already borne fruit. A recognition of the power of complement analysis has brought forward new testing but also realization of the importance of post-draw specimen handling to limit *ex vivo* activation, as well as the sometimes large difference between testing laboratory results. The increased usefulness of complement analysis and efforts to standardize and expand it means the future is strong for complement analysis.

1. Changing landscape of complement testing: growing clinical importance

The complement system is a set of proteins involved in the interconnected cascade of pro-enzymes, regulatory proteins, recognition molecules, signing molecules and receptors. Complement was first described in the late 1800's, but the list of components continues to grow. The system is composed of more than 30 proteins and has long been known for its ability to kill invading microbes at first exposure. As critical part of the innate immune system, differentiation of self from non-self involves important tagging of self and control of complement on host surfaces (Zipfel and Skerka, 2009; Ricklin et al., 2010).

For decades the primary, and nearly exclusive, use of complement testing was to test for primary immunodeficiencies or to define disease activity in systemic autoimmune diseases, focusing on a limited number of rheumatological or nephrology disorders (Ricklin et al., 2010; Skattum et al., 2011; de Cordoba et al., 2012; Holers, 2014). Only a small number of tests were used, mainly to assess total complement function, C3 and C4. While specificity was important, not a great deal of sensitivity was needed. With the abundance of components of complement in circulation, C3 being the highest at 1 to 1.5 mg/mL, the presence or absence of the proteins in the cascade could be measured by relatively simple methods (Morley and Walport, 2000). However, the field has changed dramatically due to significant developments in complement science. First, there has been a notable increase in the number of diseases and disorders recognized to be closely associated (driven by) complement (Table 1) (Thurman and Holers, 2006; Hajishengallis et al., 2017). The diseases now recognized to connect to complement are anatomically diverse, ranging from the kidney to the eye, from the rare atypical hemolytic uremic syndrome to common disorders like age-related macular degeneration. The pathophysiology of these disorders is often driven by the strong pro-inflammatory properties of complement which can affect so many properties of biology in so many tissues and organ systems. That can, in turn, be traced back to the original task of complement which is to fight infectious invaders. As a first line of defense, complement has a powerful potential to tag and even destroy invading microbes while activating the larger immune system to clear the potential damage (Walport, 2001, Skattum et al., 2011). These are functions that can be very damaging if turned on the host tissues. Many of the complement-connected disorders are, unsurprisingly, associated with an inappropriate over-activation of complement or with a failure to control complement. Some of these activations or losses of control, such as seen for mutations in complement Factor H, can be far more subtle than the yes or no

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Table 1
Complement – associated disorders.

General Disorder Category	Specific Complement Disorders
Infectious diseases	Recurrent pyogenic infections particularly Meningococci and Neisseria, haemolytic uremic syndrome (HUS),
Inflammatory disease	Rheumatoid arthritis (RA), Atherosclerosis Vasculitis, Nephritis, Systemic inflammatory reaction syndrome (SIRS), Sepsis, Ischemia/ Reperfusion injury (I/R injury), Crohn's disease,
Autoimmune disease	SLE, Multiple sclerosis, Acute myasthemia gravis (AMI), Psoriasis, Paroxysmal nocturnal hemoglobinuria (PNH)
Diseases of complement dysregulation	Atypical haemolytic uremic syndrome (aHUS), Glomerulonephritis (GN), Hereditary angioedema (HAE),
Neuro-degenerative diseases	Alzheimer's disease (AD), Age-related macular degeneration (AMD)
Others	Transplant rejection, Stroke, Myocardial infarction, Trauma, Burn, Capillary leak syndrome, Biomaterials incompatibility (Dialysis, Cardiopulmonary bypass, Plasmapheresis, etc.)

deficiency historically diagnosed by a clinical lab (Jozsi et al., 2015; Medjeral-Thomas and Pickering, 2016, Parente et al., 2017). The mutations can involve subtle changes in function, changes limited to circulation or to those on a cell surface. Detecting these more nuanced alterations in complement requires a more nuanced testing in the diagnostic laboratory.

The second event that has changed the landscape for complement analysis was the advent of therapeutics that directly target complement (Ricklin et al., 2017). Starting in 2007, the first complement-specific therapeutics entered the market. With this there is a need to not only diagnose the disease but also follow the treatment. This changed the analysis from needing to show only if a complement component were deficient to needing to know if the function was suppressed to 10% of normal, for example (Prohaszka et al., 2016). This led to the development of new assays and to the appreciation of new values in existing, but formerly rare or esoteric, tests. In addition, it has helped topropel efforts to improve the standardization between laboratories and the quality of complement testing overall. Consequently, existing laboratories specializing in complement have improved, plus there is now an increase in complement testing in the more general, large laboratories. This produces more pressure for the tests to be robust and reliable (Mollnes et al., 2007).

2. Importance of complement functional testing

Some of the earliest testing performed for complement was functional testing, and the utility and interest in the analysis of function of complement remains (Prohaszka et al., 2016). In one functional assay it is possible to test for the presence and functionality of all the components of an activation pathway and the terminal pathway at once (Fig. 1A). This ability to broadly test for complement function of a number of components of the cascade has led to an assay being referred to as a "Total Complement Activity" test, but it generally references the classical pathway functional assays. Functional complement testing proved very useful in screening for an immunodeficiency or complement activation with consumption. For immunodeficiency testing the more common classical pathway activity test (also called 'CH50') is combined with an alternative pathway activity (or AH50) assay to quickly determine where in the cascade system a deficiency lies; classical, alternative, or the shared terminal (Fig. 1A). This dramatically reduces the work required to determine the specific component deficiency. On the activation side, if a patient has an ongoing complement consumption depletion of available complement is reflected in a decreased measurable function. This has proved useful for testing for flares in autoimmune disease (Spronk et al., 1995; Leffler et al., 2014). This utility has been the prime driver in the development of the now multiple methods for testing complement function. The types of complement functional analysis largely fall into three categories, each with their own benefits and disadvantages. What these tests share is a need to first activate the specific pathway of interest, then measure the formation of the terminal complement complex in solution or by functional outcome. Defined buffer components, or the addition of inhibitory antibodies, are used to keep the other activation pathways in check. For example, the inclusion of calcium chelators (e.g. EDTA) inhibits the classical pathway through destablization of the C1 complex (Eagle and Brewer, 1929; Kabat and Mayer, 1961). The most historic form of complement function testing is what is referred to as a hemolytic test. In this method, an animal red blood cell (RBC), generally a sheep RBC, is coated with antibodies (hemolysin) making it an optimal target for classical pathway recognition. This recognition leads to activation which then results in the formation of the membrane attack complex (MAC) on the surface of the red blood cell. The MAC leads to lysis of the RBC and release into solution of the hemoglobin (Kabat and Mayer, 1961). The hemoglobin is easily quantitated by spectrophotometry which can then be related back to the percentage of RBC's that were lysed and the functionality of complement to do the lysing. As this is a sequential cascade involving the whole pathway, the relation between the concentration of serum and the amount of lysis is not linear. It is instead more sigmoidal, following the von Krug equations (Jackson et al., 1970). Therefore, the traditional method for running a hemolytic assay was to run a five-point serial dilution of serum or plasma, then use the three points that form the most linear portion of the curve that covers the mid-point of lysis, where half the RBC are lysed. From there the dilution that would lead to lysis of exactly 50% of the RBC is calculated, and the result reported as the reciprocal of that dilution (Kabat and Mayer, 1961). Some of the newer methods instead will report results as percentage of a normal or standard value. For the hemolytic method it is the relation to the report of the 50% lysis point that gives the test the abbreviated CH50 for the classical pathway assay. A similar assay, the AH50, can be performed where the target RBC is of rabbit, guinea pig or chicken origin as, unlike sheep RBCs, these are activating surfaces for the alternative pathway. Care should be taken if using these assays to screen for properdin deficiency as they all do not perform equally (Kirschfink and Mollnes, 2003). For the alternative pathway assays, classical pathway is kept inactive by chelating calcium (e.g. by EGTA) necessary to maintain the C1 complex. These hemolytic assays have the advantage of having the greatest sensitivity at the low end of function. Since they are also so complex, however, a specialized laboratory is required. Because they rely on live cells, there is the potential for variability in supply that needs to be very carefully controlled.

The most common method nowadays used in US clinical laboratories for measuring total complement is based on lysis of a liposome. In this assay a synthetic liposome stands in for the RBC of the hemolytic assay. The liposome is loaded with an enzyme, such as glucose-6-phosphate, that is easily measured on a common clinical laboratory chemistry analyzer (Frazer-Abel et al., 2016). As for the CH50, the liposome is coated with antibody to activate the classical pathway, one concentration of serum or plasma is then used in the reaction mixture and the amount of enzyme release is measured. This assay is very well suited to a large hospital-type laboratory, as it is automated with high throughput, thus fairly inexpensive to run. This testing has proven very useful when a fast yes or no answer is needed, but there is evidence that this type of assay is not sufficiently sensitive for monitoring nuanced changes at either end of the functional spectrum (Gatault et al., 2015). It is important to keep this in mind when measuring low levels of