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Pearls of Laboratory Medicine
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TITLE: Basics of Complement Testing

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Slide 1
Hello, my name is Maria Willrich. I am Co-Director of the Protein Immunology Laboratory and an Assistant Professor in the Department of Laboratory Medicine and Pathology at the Mayo Clinic.

The complement system is one of the cornerstones of the innate immune response. Complement participates in innumerous autoimmune and infectious processes. Although discovered back in the 19th century, testing for complement abnormalities has regained interest in the last decade.

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The objectives of this Pearl of Laboratory Medicine include a brief overview of the complement pathways, pre-analytical challenges for common analytes tested in the laboratory, analytical methods, and post-analytical challenges related to complement assays.

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The complement system is an important part of innate immune response, and it is also considered to have major effector mechanisms in humoral immunity.

The complement system consists of a group of proteins that are activated in sequential steps, similarly to the coagulation cascade. The complement system is activated by the recognition of cleaved complement proteins by receptors on leukocytes and vascular cells.

Primary functions of the complement system include host defense, with the activation of the inflammatory response, opsonization of microorganisms for phagocytosis and killing, agglutination of pathogens, and cell lysis of susceptible organisms.

Complement also provides a bridge between innate and adaptive immune responses through receptors on lymphocytes and antigen-presenting cells.

There are three pathways that are responsible for activation of the complement system and they differ in their mechanism of activation.

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Complement is activated in response to different threats. Complement proteins are produced mainly in the liver, but also by macrophages, monocytes, and epithelial cells in the urogenital and gastrointestinal tracts. For each of the pathways, the recognition molecule that will trigger activation of complement is different, and so are some of the proteins involved in each of the cascades.

The classical pathway is activated by an antibody binding to a target antigen, forming a complex. IgM has the greatest ability to activate complement among all immunoglobulins. C-reactive protein can also activate the classical pathway.

The alternative pathway does not require an immune complex for it to be activated. There is a surveillance role for the alternative pathway, mediated by continuous hydrolysis of one complement component called C3. Therefore, the alternative pathway is always active at low levels. Activating surfaces include pathogens and particles of microbial origin, including polysaccharides, lipopolysaccharides, and glycoproteins, as well as non-pathogens such as certain heterologous red blood cells.

The lectin pathway is analogous to the classical pathway, except that the triggers for its activation are bacteria containing mannose on its cellular membranes.

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This slide illustrates how the various components within the complement system all interact. In the classical pathway, an immune complex formation exposes sites in the Fc fraction of immunoglobulins that will allow attachment of C1q, which is the first subcomponent of complement. C1q is complexed to C1r and C1s proenzymes to form C1. C1 is a large calcium dependent complex that when bound to an immune complex will initiate the process that leads to cleavage of C4 and C2.

Before continuing to the C3 convertase, we will look at the alternative pathway in the center of the slide. In the alternative pathway, lipopolysaccharides (or LPS) from Gram negative bacteria, fungi, and yeast cell walls or cobra venom may start the cascade, as will other molecules. In addition, spontaneous activation from hydrolysis of C3 occurs and generates C3b. C3b, a split product, will bind to factor B; which will be cleaved by Factor D to form a fluid phase C3 convertase. This entire process is called the C3 amplification loop. Properdin bound to microorganisms such as Neisseria or damaged cells can recruit C3b and activate the alternative pathway as well.

For the lectin pathway, bacteria containing mannose are identified by the recognition molecule mannose binding lectin (MBL) and its associated proteases, mannose associated proteases 1 and 2 (MASP 1 and 2), both of which are structurally very similar to the C1 complex formed in the classical pathway. MBL and MASPs drive cleavage of complement proteins initiating the cascade with cleavage of C4 and C2, and from that point on, the lectin cascade is identical to the classical pathway.

Ultimately, all pathways lead to formation of a stable C3 convertase. Once there is enough C3b deposition to interact with the C3 convertase, the C5 convertase is formed. C5 split products will either act as anaphylotoxins (C5a) or generate the ultimate product of the cascade, known as the membrane attack complex (MAC). When the MAC is on the surface of a cell membrane, it will create a pore and cell lysis *(1).*

The complement system is tightly regulated by fluid-phase inhibitors as well as membrane bound regulatory proteins. There are three primary levels of control which include the initiation step of the classical and lectin pathways, generation of the C3 and C5 convertases, and the assembly of the MAC.

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Although tightly regulated, there are conditions where complement is dysregulated and it may be due to either genetic abnormalities or acquired conditions.

C1 esterase inhibitor deficiency is a heterozygous inherited genetic deficiency known as the basis for hereditary angioedema, a condition with recurrent attacks of subcutaneous or submucosal edema.

Complete complement deficiencies are rare, with a combined prevalence of 0.03% in the general population. Inheritance is autosomal and expression is co-dominant. Common clinical presentations include increased susceptibility to Neisserial infections, encapsulated microorganisms, and systemic autoimmune disease.

For C1, C2, and C4 deficiencies, there is an inability of immune complexes to activate the classical and lectin pathways. When there is deficiency of C5, C6, C7, C8, and C9, patients are unable to form the membrane attack complex. Deficiencies in complement factors that start the cascades are usually more severe whereas deficiencies in the terminal complement components are milder, and often, individuals are healthy. For more detailed manifestations of the complement deficiencies and dysregulation, please refer to the Pearl of Laboratory Medicine on “Clinical Applications of Complement Testing” by David Murray (www.traineecouncil.org).

Allele variants affecting C3 convertase regulation in the alternative pathway have also been described. That is the case for C3 itself, CD46, Factor H, Factor I, as well as Factor B.

Acquired complement dysregulation is comprised of a more heterogeneous group of disorders. In autoimmune disease, overactivation of the classical pathway due to the excess of immune complexes may result in tissue deposition of the immune complexes, particularly in the kidneys. Decreased complement receptor 1 and complement receptor 2 on the surface of erythrocytes can be an acquired deficiency as a result of immune complex clearance.

Paroxysmal Nocturnal Hemoglobinuria (PNH) is the result of the deficiency of 2 glycosylphosphatidylinositol (GPI)-anchored proteins, Decay Accelerating Factor (DAF) or CD55 and CD59, which are complement inhibitors. The lack of complement inhibitors leads to continuous activation of the alternative pathway. PNH is characterized by complement mediated intravascular hemolysis, occasional hemoglobinuria, and venous thrombosis.

Autoantibodies against known regulators of the Alternative Pathway (Factor B, Factor H, Factor I, and C3 convertase) have been described and may also result in continuous activation of the cascade. Excess C3 is a common byproduct of this undesired amplification loop and may cause deposition of C3 in tissues such as the kidneys which leads to extensive damage.

It is also important to note that tissue injury may result from deposition of a combination of immune complexes and complement factors, after classical pathway activation, or only complement factors, when the alternative pathway is dysregulated.

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This figure also illustrates the classical and alternative pathways, although I would like to focus attention to the regulators of the cascades, beginning with C1 esterase inhibitor in the classical pathway on the left, then C4 binding protein, then complement Factor H as an inhibitor of the C3 amplification loop *(2).*

For the alternative pathway, on the top right of the slide, Factors B and D are activators to generate the C3 convertase, whereas Factors H and I are inhibitors of the C3 lysis.

And last but not least, vitronectin and clusterin are inhibitors of the membrane attack complex, keeping the complex soluble and avoiding pore formation on cell membranes *(3).*

This picture illustrates and highlights just how many stages there are in the pathways where dysregulation can occur. Mutations generating defective proteins are depicted by broken red boxes and autoantibodies to complement factors shown as orange immunoglobulins.

Slide 8:
Now that we have reviewed the fundamental background of the complement system, we can review what the appropriate indications are to order testing to evaluate complement function or abnormalities. Testing is indicated when there is suspicion for primary immunodeficiency, presentation of angioedema *(4)*, recurrent pyogenic infections, autoimmune diseases such as systemic lupus erythematosus (SLE), or a family history of complement abnormalities.

Testing may also be considered if there are signs of tissue injury potentially mediated by complement, such as in glomerulonephritis, graft rejection, sepsis, unexplained intra- or extravascular hemolysis, Hemolytic Uremic Syndromes, or Paroxysmal Nocturnal Hemoglobinuria *(5).*

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Several complement assays are available. Genetic tests may be performed as targeted mutation analysis or next generation sequencing panels. Multiple genes may be studied but frequently include: C3, CD46 or membrane cofactor protein (MCP), complement Factor B (CFB), complement Factor H (CFH) and its related proteins 1 through 5 (CFHR1-5), and complement Factor I (CFI) *(6).* Detailed discussion about these assays is beyond the scope of this presentation. Instead, I will focus from this point forward on serologic complement tests.

Serologic tests for complement exist within three main categories:

1. Measurement of total complement function or activity
2. Complement factors, individual antigen concentrations
3. Detection of autoantibodies against complement factors

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Before the analytical methods for serologic assays are discussed, there are a significant number of pre-analytical issues laboratorians must address. One of the primary challenges is to stop complement activation in vitro following specimen collection.

Complement proteins will adhere to cell membranes and they are biologically designed to interact with immunoglobulins. Moreover, complement proteins are heat labile, and exposure of the sample to room temperature causes degradation of complement components. Poor sample stability is a major challenge which is not easily overcome.

The alternative pathway is constantly active at low levels and *in vitro* activation may result in a consumptive process of complement factors that is extremely difficult to contain once the C3 amplification loop has been started. Clotting is also associated with complement activation and therefore, consumption of the early components of the complement pathways and for some assays, plasma is preferred rather than serum samples. Both degradation by temperature and consumption of complement factors after activation will lead to falsely low results. It is well accepted and recommended to freeze specimens immediately after collection. Long term stability is optimal when the sample is kept at -70 degrees Celsius prior to testing *(7).*

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Common analytes measured within the classical pathway include:

- CH50 or Total Complement function
- C1q, C2, C4 individual components (Functional and Antigen)
- C1q esterase inhibitor (Functional and Antigen)

To evaluate the alternative pathway:

- AH50 or Alternative Pathway Function
- Factor H (Antigen) and autoantibodies against Factor H
- Factor B (Antigen) and split products

Within the terminal Pathway:

- C3 (Functional and Antigen)
- C5-C9 (Functional and Antigen)
- Soluble MAC (sC5b-9 or sMAC)
- C3 Nephritic Factors (autoantibodies against C3 convertase)

Within the lectin pathway:

- Mannose-Binding Lectin (Functional and Antigen)

CH50 and AH50 are the most appropriate primary assays used as screening methods for complement abnormalities. Abnormal results in one or another or both will help direct further testing.

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There are automated and manual methods available which can be used to quantitate the concentration of complement factors. These assays measure the amount of antigen in the sample, and are most commonly reported in milligrams or micrograms per deciliter.

Nephelometry methods quantitate based on the light scatter from immune-complexes, and are used to measure C1q and C1q esterase inhibitor, C3, C4, and C5, Factor B, and Factor H.

For assays where finding specific antibodies is challenging, manual methods such as radial immunodiffusion are still considered the standard of practice, particularly for C2.

ELISAs are another common method; as an example, ELISA is often used to test for mannose-binding lectin or autoantibodies against Factor H or Factor I.

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Nephelometry is a widely used technique based on the physical property of light scattering of immune complexes. For complement factor testing, it is used to measure the antigen concentrations or complement quantitation.

The intensity of light scatter generated by an immune complex is proportional to the amount of complexes present in the sample. In some assays, latex beads are used to drive scatter towards a higher yield signal. The method will not be discussed in detail during this presentation but importantly it should be mentioned that particles, solvent, and macromolecules all scatter light, which means lipoproteins and chylomicrons become problematic interferents. Lipemic samples can generate signal above the background despite lack of antigens of interest and therefore, cannot be tested. It is not common practice in the field to ultracentrifuge the specimens to remove lipemia because of the poor stability of the samples and potential risk of complement activation.

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Radial Immunodiffusion is a versatile method that has been in practice for decades, since the 1960s.

RID uses an agarose gel containing either antiserum to a given complement factor or heterologous red blood cells from sheep, rabbit or chicken.

For both forms of the assay, wells are punched into gel, and samples and standards allowed to diffuse.

If it is an antiserum type radial immunodiffusion, a precipitin ring will be observed. If it is a red blood cells agarose gel, hemolysis will be observed around the well. In both cases, the diameter around the well is proportional to either the antigen concentration or the complement activity.

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The advantages of radial immunodiffusion for laboratory testing include the fact that it is a simple and versatile technique, allowing for measurement of antigen concentrations or complement function. It does not require sophisticated instrumentation and uses a small volume of sample for testing. Disadvantages or challenges are exemplified by the manual and labor intensive techniques which are affected by variations in temperature and time of incubation. Those variations may also create artifacts and interpretation is subjective. It also takes a long time to perform the assay since incubation time is often several hours, which means the reporting turnaround time is at least a couple of days.

Radial immunodiffusion has been largely replaced by more sensitive and automated methods such as nephelometry and ELISAs.

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The main methodology to study complement system function is based on hemolytic assays. They form the initial approach to complement testing, and are often used as screening tests. These assays - applied to CH50 and AH50 - use red blood cells and evaluate cell lysis, which is the end-product of the complement activation.

To assess total complement function, or CH50, sheep erythrocytes are sensitized with rabbit antibody. The dilution of patient's serum needed to lyse 50% of erythrocytes is then determined. The assay requires all proteins of the classical and terminal pathways. Deficiency in C1 or C2-C8 will result in little or no lysis to the red blood cells, with hemolysis less than 5%. C9 deficient patients may have residual CH50 function, with less than 30% of RBCs hemolyzed.

To assess alternative pathway function, or AH50, a buffer is used to block the classical pathway activation. Rabbit erythrocytes are then used to spontaneously activate the alternative pathway (some heterologous red blood cells are known to activate the alternative pathway of complement). The assay requires all components in the alternative pathway and terminal pathway. Little or no lysis is observed for deficiency of C3-C9, Factor D, and Properdin.

C3 is the most abundant complement factor; deficiencies of Factor H, Factor I, or the presence of C3 nephritic factors will often cause low results in both assays.

An alternative to the old-fashioned hemolytic assays are ELISA assays, which measure neoepitopes generated after complement activation. ELISAs are becoming popular in this field because they are largely amenable to automation; however, they are still considered laboratory-developed tests.

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Automated methods for complement function analysis include a liposome enzymatic assay and an ELISA which measures neoepitopes of the soluble membrane attack complex, represented here by panels A and B, respectively.

Briefly, for the liposome enzymatic assay illustrated in panel A, the total complement lytic activity is measured by mixing patient serum in the presence of immune complexes created by the reagents. Liposomes containing glucose-6-phosphate (G6P) will be lysed if the classical and terminal complement pathways are activated. G6P is then exposed and an enzymatic reaction using NAD can be measured on several automated platforms, with absorbance measured at 340 nm.

Panel B illustrates a more simple and conventional ELISA method for measurement of the alternative pathway function. With lipopolysaccharides from Gram negative bacteria coated on a solid-phase support or ELISA plate, the alternative pathway in patient's serum will be activated to generate the C5b-C9 complex, or the membrane attack complex, MAC. The conjugate antibody targets neoepitopes generated after the formation of the MAC, making this a functional assay. Similar ELISA approaches are available for the classical and lectin pathways.

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In the clinical laboratory, proficiency testing is a mandatory quality assurance activity for all analytes. However, the availability of external commercial programs and materials is limited. A majority of laboratories implement alternative assessment of performance for proficiency testing using blinded or split samples exchanged between institutions. There are still shortcomings because the assays are not standardized or harmonized, which means the reference intervals and performance between methods is often not interchangeable across laboratories.

Post-analytical interpretation of results requires review of results from multiple tests, ideally in a panel format. Several tests are needed for a comprehensive overview of complement function due to the complexity of testing and relationship to clinical diseases.

Poor specimen stability results in poor reproducibility of the complement assays. Eculizumab, a monoclonal antibody against C5, is used for the treatment of atypical HUS and paroxysmal nocturnal hemoglobinuria (8-10); if the patient is on eculizumab, it will impact the test results.

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In my closing remarks, I would like to reiterate that complement is a complex system of highly regulated proteins. Multiple tests are needed for a comprehensive overview of the cascade. Isolated complement measurements are rarely useful and should be taken in the context of the patient's clinical presentation.

Evaluation of complement has utility in a variety of clinical circumstances. Functional assays should be the first tier of testing, followed by antigen quantitation.

Strong laboratory oversight is required due to the highly complex nature of the entire testing process and interpretation of results.

Slide 20: References

Slide 21: Disclosures

Slide 22: Thank You from www.TraineeCouncil.org
Thank you for joining me on this Pearl of Laboratory Medicine on "Basics of Complement Testing."