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TITLE: Clinical Applications of Complement Testing

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Slide 1: Introduction

Hello, my name is David Murray. I am Co-Director of the Protein Immunology Lab at the Mayo Clinic. Welcome to this Pearl of Laboratory Medicine on "Clinical Applications of Complement Testing."

There has been renewed interest in clinical complement testing within the last couple of years. This renewed interest has been driven primarily by the availability of new monoclonal therapeutics which are capable of blocking the complement system. Although many of these drugs are in various stages of development, FDA approval of eculizumab (a C5 inhibitor) for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and complement-mediated hemolytic uremic syndrome (also known as atypical HUS) is reviving interest in testing for complement system defects.

These therapeutics are quite costly; hence, an accurate diagnosis of complement-related diseases and defects is critical for proper utilization of these drugs and effective use of health care dollars. My experience as a lab director overseeing complement testing has taught me that complement tests are poorly understood. It is not uncommon for our lab to see orders for very specific complement tests without evidence of a complement defect in the screening assays. As future lab directors overseeing reference lab tests, you will be faced with guiding clinicians on ordering these tests. The goal of this Pearl is to aid you in directing clinicians who are attempting to evaluate the complement system.

Slide 2: Learning Objectives

I have three learning objectives for this Pearl:

1. To enable the listener to describe and categorize the diseases for which complement testing is warranted.
2. To allow an appreciation for the different roles of complement functional testing and complement protein quantitation.
3. To increase your ability to guide clinicians on the appropriate selection of complement tests.

Slide 3: What is the Normal Role of the Complement System?

Before we discuss the diseases and clinical testing related to the complement system, it is important to ask or remind ourselves: What is the normal role of the complement system?

The complement system has many functions and although this slide somewhat oversimplifies things, these two categories will suffice for this presentation. Some have compared the complement system to the army of the body, destroying foreign enemies and cleaning up at the end of the battle.

Complement is one component of the innate immune system. Its primary role is in the clearance of microorganisms and the neutralization of viruses. The split products generated from the complement protein cascade also serve as chemotactic agents, bringing inflammatory cells into the site of infection and stimulating vasodilatation and permeability to allow the inflammatory cells to the site of infection. Defects in this function of the complement system lead to increased infections while defects in the regulation of this function lead to unnecessary destruction of cells within the body.

Second, the complement system is involved in the clean-up of the cellular damage caused by the immune system's response to infection. Defects in this function can lead to immune complex diseases such as lupus.

Interestingly, symptoms associated with inherited complement defects often do not manifest until late childhood or early adulthood. Many individuals can live normally with complement defects until some triggering event occurs. This incident could be an infection, pregnancy, or some other injury which activates the complement system and thereby exposes the defect in the system.

Slide 4: Diseases Related to Complement Testing

Moving on to the diseases associated with complement testing, I like to think of the two extremes of defects - low activity and unregulated or "over"-activity.

If a person has an inherently low complement activity, they typically present with either recurrent infections or with early onset autoimmune disease. Defects in formation of the membrane attack complex (or MAC) greatly hampers the ability of the immune system to clear pathogens such as Neisseria. Therefore, complement testing is warranted in the workup of patients with suspected immunodeficiencies. In addition, inability of the complement system to deal with clearance of immune complexes can result in early onset systemic lupus erythematosus (SLE)-like symptoms.

On the other hand, a patient with unregulated complement typically has symptoms associated with endogenous cellular destruction or, as in the case of angioedema, an increase in vaso-activity from the overproduction of complement split products. As an example, normal red blood cells contain complement inhibitors in their cell membranes. If the inhibitor is lost from the red blood cell surface, the red cells will inadvertently be destroyed by the complement system, giving rise to the hemoglobinuria characteristically associated in patients with PNH. Also, an unregulated alternative pathway can have adverse effects on the endothelial cells of the kidney,

resulting in a glomerulonephritis seen in atypical HUS, dense deposit disease, and C3-glomerulonephritis. We will discuss these conditions in later slides.

Slide 5: Complement: A Well-Regulated System

This diagram displays the three arms of the complement system: the classical pathway, the lectin pathway, and the alternative pathway.

Each pathway has its own initiator and regulators but all three pathways converge at C3 and result in the formation of the membrane attack complex (MAC). Formation of the MAC on a target such as bacteria results in loss of cellular content and death of the bacteria.

When thinking about complement defects and diseases, it is helpful to keep in mind the main initiators and regulators of each pathway. In doing so, it will help you link the disease mechanism to its complement pathway.

The classical pathway was the first complement pathway to be described and was found to be initiated by the aggregation of antibody antigen complexes. Therefore, it is the main pathway activated by the adaptive immune response or in immune complex disease. The main serum regulator of the classical pathway is C1 esterase inhibitor.

The lectin pathway is very similar to the classical pathway with the substitution of mannose binding protein replacing the role of C1qrs. Therefore, organisms with mannose on their cell surface will be directly neutralized by complement without immunoglobulins. Knowledge about diseases due to defects in the lectin pathway are still evolving so we will not elaborate further about this pathway in this Pearl.

The alternative pathway is unique as it is thought to be constitutively active and not dependent on immunoglobulins. Since initiators play a lesser role in this pathway, regulator proteins such as Factor I and Factor H have a more prominent role in disease states.

Having a system which can cause cellular destruction requires tight regulation to avoid “friendly fire.” This diagram also reveals the complexity of the regulation of the overall system. These regulators are present both within the serum (the proteins listed in the yellow circles) and built into the cellular surfaces (the proteins in the blue circles). Although diseases can result from the abnormal expression of regulatory proteins on the cellular surface, we will only briefly touch on these diseases and focus more on the serologic complement testing.

Abnormal laboratory complement testing can be the result of either a primary cause, meaning there is an inherent defect in the complement system or secondary to physiologically normal complement activation. The differential of a primary versus a secondary cause of complement activation is challenging. There is a large list of secondary causes of complement activation. An overwhelming bacterial infection or immune complex disease will activate the complement system and can look like a primary defect. When possible, the interpretation of the complement testing should be done in light of any clinical information available. This challenge can also cause overutilization of complement tests. We will discuss this more when talking about individual complement-associated diseases.

Slide 6: Primary Complement Defects

This slide summarizes the current state of knowledge about primary complement defects. I find this figure to be helpful and I keep a copy posted in my office.

The primary disease can be either genetic, meaning the patient was born with an abnormal allele which makes a defective protein or halts protein production, or the disease can be acquired, meaning that an autoantibody has neutralized or eliminated the complement protein. Examining this slide carefully will allow you to determine if autoantibodies or defective proteins have been documented to cause disease.

Slide 7: Eculizumab: Anti-C5 Monoclonal Antibody

Eculizumab is a new monoclonal antibody directed at neutralizing the function of the complement component C5. In doing so, the formation of the membrane attack complex is blocked. This drug has the same effect on complement laboratory tests. At therapeutic doses, the CH50 and AH50 are completely blocked resulting in low values for these tests. However, all quantitative levels of the complement factors (including C5) will be within the reference range. To properly assess the function of the complement system, it is important to draw patient specimens before administration of this drug.

Slide 8: General Overview of Primary Complement Defects

In addition to evaluating low versus high activity, I like to think about the mechanism by which the primary complement defect occurs, which is important in order to properly select the right test. I prefer using the word defect since not all “complement deficiencies” end up with low levels of the protein. In these cases, functional testing is needed to pinpoint the defect.

This table classifies the type of primary protein defect into either germline or acquired. In general, germline defects present earlier in life compared to acquired defects. Hence, children are more likely to have germline defects while adults are more likely to have acquired defects.

The second important distinction involves determining in which part of the complement cascade the protein defect exists - in the regulator proteins or in the cascade factor proteins. For the factor proteins, almost all germline defects result in immunodeficiency and most of the disease symptoms come from loss of the classical pathway function. Patients with immunodeficiency associated with the alternative pathway factor deficiencies are rare and only scattered case reports are found in the literature.

For the acquired factor protein deficiencies, autoantibodies such as C3 nephritic factor can stabilize the C3 convertase leading to renal disease, typically glomerulonephritis. The majority of complement regulator protein defects result in renal disease. The role of autoantibodies to the classical pathway factors is not well-investigated. In our lab, we have seen a patient with renal failure who had autoantibodies to C4, but the connection to disease still remains unclear.

Germline mutations in alternative pathway regulators result in renal disease and macular degeneration. Renal disease typically presents with microthrombi in the kidney and associated with low platelet counts. These are the thrombotic microangiopathies (or TMAs). Macular degeneration has a link to the alternative pathway through Factor H but testing is not

recommended since the treatment is simply don't smoke and take your vitamins - something we all should be doing.

Recently, an adult form of atypical HUS has been termed C3-glomeulonephritis (or C3GN) with renal biopsy findings showing glomeruli with C3 immunofluorescence positivity without immunoglobulin positivity.

Germline mutations in the classical pathway regulators usually involve specific symptoms. The two classic diseases are PNH and hereditary angioedema. We will talk more about these later.

Slide 9: Two Major Categories of Complement Testing

Before discussing the recommended testing in each of these diseases, I would like to review the two basic types of testing used in evaluating the complement system. These can be broken into quantitation, which measures the level of the protein, and functional tests, in which function and concentration of the protein are indirectly assessed. Functional tests can be further subdivided to include those which screen the entire cascade and those that test the function of individual components. Most clinical screening is focused towards the classical and alternative pathways. The CH50 and AH50 tests screen the function of the complement system in a similar manner as PT and PTT screen the coagulation system. The soluble membrane attack complex (or sMAC) can be used to assess for activation of the complement system. The sMAC adds value in cases for which consumption of the complement factors has not resulted in abnormal CH50 and AH50. In this sense, the sMAC has a similar role to D-dimer in the fibrinolytic system.

Given the nature of the defects for which we are testing, both quantitation and function may be necessary. For instance, in the case of a monogenetic defect in a single complement protein, results from quantification of the protein may be within the reference range but the function may be absent.

Slide 10: Pre-Analytical Variables and Specimen Collection Are Critical

Dr. Maria Willrich has prepared a Pearl titled, "Basics of Complement Testing," which discusses the analytical methods used to test the complement function. I would suggest viewing that Pearl, available at www.traineecouncil.org, for more detailed information on these tests.

If you remember only one point from this presentation, I hope it would be this point:
Pre-analytical handling of the sample is critical.

Unlike coagulation testing where calcium can be sequestered to preserve function, draw tubes with complement stabilizers are not widely available. Post-draw activation of the complement system can occur and samples should be frozen as soon as possible once cellular components are removed. Secondly, a substantial portion of the patients with suspected complement-related diseases are undergoing therapeutic apheresis or treatment with eculizumab. It is essential to obtain a sample before the induction of apheresis. The half-lives of the complement proteins are not known but we give the general recommendation that testing should be performed as far from therapeutic apheresis as possible. We also recommend abnormal results be confirmed in a second sample.

Slide 11: Testing for Complement Defects

For pediatric patients suspected of having a primary immunodeficiency without renal damage, a screening of the classical pathway function using the CH50 is a sufficient test. If values are below the reference range, it would then be advisable to look at the levels and function of each of the classical complement factors. If any of the factors are abnormally low in concentration or function, genetic panels are available that can sequence the complement factor genes.

In the adult patient, the association of immunodeficiency with complement is not common. Occasionally, however, you can have a patient present later in life. In adults, a simple CH50 test is the best place to start.

This seems to be an appropriate time to address a common question that we receive in our lab: What is the clinical significance of an elevated CH50 or AH50? There are no known disease associations with an elevated value. For the most part, it is thought that this is part of the acute phase response.

Slide 12: Testing for Complement Defects

For pediatric patients with SLE-like symptoms, in addition to complement functional testing, quantitation of the early classical components (C1q, C2, and C4) is warranted due to the high association with SLE. More than 90 percent of C1q-deficient individuals develop SLE and may also have recurrent bacterial infections. Total C4 deficiency is rare but approximately 80 percent of patients with low C4 manifest early SLE. Patients with early classical component defects have persistently low CH50 results.

In the adult SLE patient, complement testing is more for disease activity than for diagnosis. Rheumatologists will follow C3 and C4 levels to document disease flares.

Slide 13: Testing for Complement Defects

Patients who present with renal failure, low platelets, and microthrombi in small vessels (thrombotic microangiopathies or TMAs) can have disease associated with complement defects.

As with the other diseases we are discussing, the first line testing for a pediatric patient with renal disease should not be complement testing but it may be considered when other more common causes for renal disease are ruled out.

Dense Deposit Disease (DDD) and complement-mediated HUS (also known as atypical HUS) are diseases associated with alternative pathway disregulation. In younger populations, the majority of defects are due to mutations in the alternative pathway regulator proteins. To properly screen in these cases, a broader panel of tests is recommended. This broader screen is necessary to assure that the defect is isolated within the alternative pathway. For example, a low AH50 value could be due to consumption of C3 by activation of the classical pathway secondary to a bacterial infection or immune complex deposition.

The sMAC is a sensitive measurement for complement activation and can be used when complement activation has not proceeded to the point of protein consumption. The value of a normal C4 in this setting cannot be overstated. In my experience, a normal C4 value in a patient

with low AH50 really helps to isolate the problem to the alternative pathway. In our lab, we use split products specific for the classical and alternative pathways to help determine which pathway is active.

Lastly, Factor H and Factor B are often consumed in the acute phase of activation; low levels of either are suggestive of an alternative pathway consumptive process.

Slide 14: Testing for Complement Defects

In the adult patient, the major differential for TMAs is among thrombotic thrombocytopenic purpura (TTP), diarrhea-associated HUS (d-HUS), and complement-related HUS. Since both TTP and d-HUS are more prevalent than a complement-associated HUS, screening for TTP and d-HUS before complement testing is standard practice. By definition, patients with TTP have low levels of ADAMTS-13 function. Also, the d-HUS is typically the result of infections with shiga-toxin producing bacteria. Therefore, we recommend testing for the presence of shiga toxin and ADAMTS-13 function before complement testing. If both of those lab tests are negative, complement testing can then be performed to look for the more uncommon complement-associated HUS.

The screening panel is the same as we just described for the pediatric patient and should include measurement of the alternative and classical pathways (AH50 and CH50), C3, C4, Factor H, and Factor B. It is also beneficial to look at activation markers for complement. We typically use the sMAC as a general complement screen, CD4 as a marker of classical pathway activation, and Bb as a marker of the alternative pathway.

TTP is considered a medical emergency and thus, patients with suspected TTP are sent to apheresis quickly. It is important to collect specimens prior to this procedure since fresh frozen plasma will be given to the patient after the procedure. This is often overlooked when ordering tests and makes interpretation of the results of complement testing ambiguous.

Slide 15: Classical Atypical-HUS Findings

A few small studies have been reported in the literature reviewing the results of complement testing on patients with TTP, d-HUS, and complement-mediated a-HUS. This slide summarizes the classic lab results obtained during active disease, which gives the highest specificity for diagnosis of a-HUS. The classic findings are low AH50 activity, low Factor H and/or B protein levels, normal C4 protein levels, and elevated activation markers.

Slide 16: Follow-up Testing for Alternative Pathway Defects

If an alternative pathway defect is documented, follow-up testing to isolate the cause of the abnormality should be undertaken. Currently, the three most common tests considered are antibodies to factor H, C3 nephritic factor, and gene panels sequencing complement proteins and inhibitors. C3 nephritic factor is thought of as an autoantibody which stabilizes the C3 convertase resulting in prolonged alternative pathway activation. These tests are available at select reference labs.

Slide 17: Testing for Complement Defects

Patients who have recurrent angioedema or swelling without hives or itching may have acquired or hereditary angioedema with uncontrolled classical pathway activation. The fundamental underlying defect is in the C1-esterase inhibitor function. Screening in these patients requires closely evaluating the classical pathway. By definition, C4 antigen levels are always reduced during the acute phase of the symptoms. C1INH level and C1INH function are helpful in differentiating between the acquired and hereditary form. It is important that the patient be off treatment and the optimal time for testing is during the time of attack and following recovery. If the level and function of C1INH are both low and the patient is younger than 40, the underlying defect is most likely genetic. If the patient is over the age of 40 and the level is low, acquired angioedema is the most probable diagnosis.

Slide 18: Testing for Complement Defects

Paroxysmal Nocturnal Hemoglobinuria (PNH) is disease associated with loss of complement inhibitors CD59 and CD55 on the cell membranes of red cells. In the absence of these inhibitors, complement proteins that bind mammalian cell membranes (self) through the alternative complement pathway can lyse self-cells as if they were bacteria. This results in the classical presentation of early morning cola-colored urine. Flow cytometry is the most useful and accepted method to confirm the diagnosis of PNH. There is not a role for testing serum complement components to diagnose this disease.

Slide 19: Summary

In conclusion, I would like to remind you of the major points in this Pearl. As a lab director, you will likely get questions about the complement system testing since it is rarely or poorly taught in most medical schools. Remember that complement abnormalities are relatively rare and thus, careful review of complement orders will be beneficial both to the patient and to your institution. I suggest having a diagram of the complement system posted in your office similar to Slide 6 in which the defects and the disease are labeled. Lastly, one of the most overlooked aspects of complement testing is sample collection. Proper handling of the specimen is KEY to getting accurate results and good patient care, as the patient will need to be redrawn if the sample is not collected or handled properly.

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 “Clinical Applications of Complement Testing.”