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Answers in Life Science

THE COMPLEMENT SYSTEM

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- CLINICAL MANIFESTATIONS
- DEFICIENCY AND DYSREGULATION
- COMPLEMENT TESTS
- THERAPEUTIC MONITORING
- HANDLING OF SAMPLES

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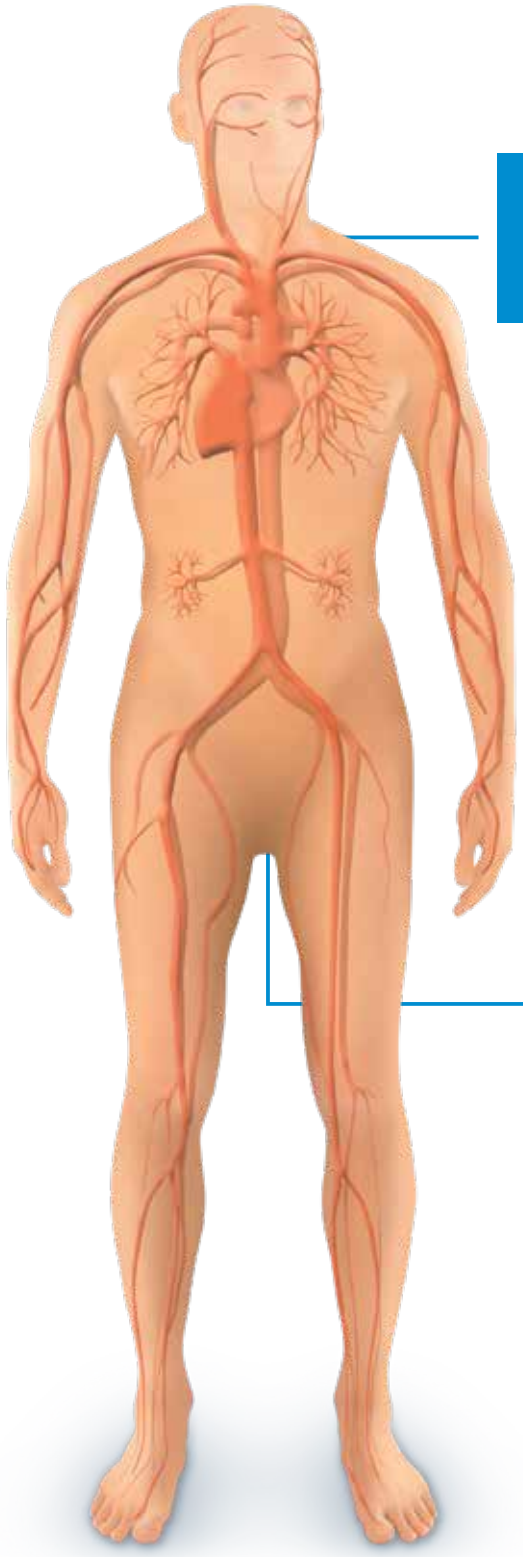

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1. THE COMPLEMENT SYSTEM

1.1 STRUCTURE AND MOLECULAR FUNCTION

The complement system is part of the host defense with a number of biological effects, many of which contribute to the inflammatory reaction mainly by activation of cells like leukocytes and endothelial cells. An intact complement system is required for protection against infection and for maintaining internal tissue homeostasis. However, the system is a double-edged sword because improper, enhanced, or uncontrolled activation is disadvantageous and potentially harmful for the host. Complement comprises more than 30 proteins acting together in a highly specific manner and is kept under strict control by regulatory proteins. The system can be activated by three initial pathways (Figure 1).

The classical pathway is typically activated when natural or elicited antibodies bind to antigen, but it can also by activated independent of antibodies by a number of substances, like C-reactive protein (CRP), when bound to the surface of a cell. C1q triggers the serine proteases C1r and C1s, the latter cleaving C4 to C4b, which exposes a specific binding site for C2. C1s then cleaves C2, and the resulting C3 convertase C4b2a cleaves C3 to C3b to form the C5 convertase C4b2a3b. Splitting of C5 to the highly potent anaphylatoxin C5a and the C6-binding fragment C5b is the last enzymatic step in the cascade.

Activation of the lectin pathway is initiated by mannose-binding lectin (MBL), ficolins 1–3, and several collectins, all of which recognize conserved structural patterns on microbes and on various structures on damaged self cells. MBL is homologous to C1q and triggers three MBL-associated serine proteases (MASPs), of which MASP-1 and MASP-2 contribute in further downstream complement activation. Subsequent lectin pathway steps are virtually identical to classical pathway activation by forming the same C3 and C5 convertases.

The alternative pathway mechanism differs from the classical and lectin pathways. Under normal physiological conditions, the C3 molecule undergoes a low-grade spontaneous hydrolysis of the internal thiol-ester and thereby binds factor B, which is cleaved by factor D to form a C3 convertase containing the whole C3 molecule ($C3(H_2O)Bb$). This complex then cleaves C3 to C3a and C3b. The latter binds factor B, which is cleaved by factor D, and the second alternative pathway C3 convertase C3bBb is formed. Properdin, the only regulator of complement that amplifies activation, binds to C3bBb and stabilizes this complex, which then cleaves C3 and binds to C3b to form the C5 convertase $C3b_2BbP$ that cleaves C5 in the same manner as the classical/lectin pathway C5 convertase. A major function of the alternative pathway is to amplify classical and lectin pathway activation from the C3 stage. The terminal pathway proceeds in the same way irrespective of the initial pathway activation by assembly of the C5b6 complex and subsequent binding of C7 to form an amphiphilic complex that is able to insert into a lipid membrane. One C5b-7 moiety binds one C8 and one or more C9 molecules thus forming the terminal C5b-9 complex (TCC). If this occurs on a membrane, it inserts as the C5b-9 membrane attack complex (MAC), which creates a physical pore that penetrates the membrane and leads to transmembrane leakage and subsequent cell or bacterial lysis. Notably, if the attack is in a sub-lytic dose, it might activate the cell and induce a subsequent inflammatory response. If the activation occurs in the fluid phase and there is no membrane present, the C5b-7 complex binds to vitronectin and clusterin (both of which are fluid-phase regulators of the terminal pathway) and the final assembly of C8 and C9 retains their hydrophilic properties and the soluble form of TCC, sC5b-9, is formed. This complex is non-lytic and to our knowledge does no harm, but it is a very useful marker for the degree of complement activation in plasma and other body fluids.

Complement activation is strictly regulated by inhibitory control proteins (figure 1). In the fluid phase, C1-inhibitor (C1-INH) controls C1r, C1s, and MASPs, whereas carboxypeptidase N inactivates the anaphylatoxins C5a and C3a by cleaving off the terminal arginine. Factor I cleaves and inactivates C4b and C3b and uses the soluble co-factors C4b-binding protein (C4BP) in the classical/lectin pathway and factor H in the alternative pathway. The membrane regulators complement receptor 1 (CR1; CD35), membrane co-factor protein (MCP; CD46), and decay accelerating factor (DAF; CD55) regulate complement activation by either acting as co-factors for factor I-mediated cleavage of C4b and C3b (CR1 and MCP) or accelerating the decay of the C3 and C5 convertases (CR1 and DAF).

CD59, also a membrane-bound regulator, prevents the binding of C9 to the C5b-8 complex in the terminal pathway. CR1 and MCP are transmembrane proteins, whereas DAF and CD59 attach to the cell membrane via a glycosylphosphatidyl-inositol anchor. Many of the biological effects induced by complement activation are mediated by membrane receptors such as receptors for C3a (C3aR), C5a (C5aR1 and C5aR2), C3b/C4b (CR1), iC3b (CR3; CD11b/ CD18 and CR4; CD11c/ CD18), and C3d (CD21), the latter tightly linking the complement system to the adaptive immune system.

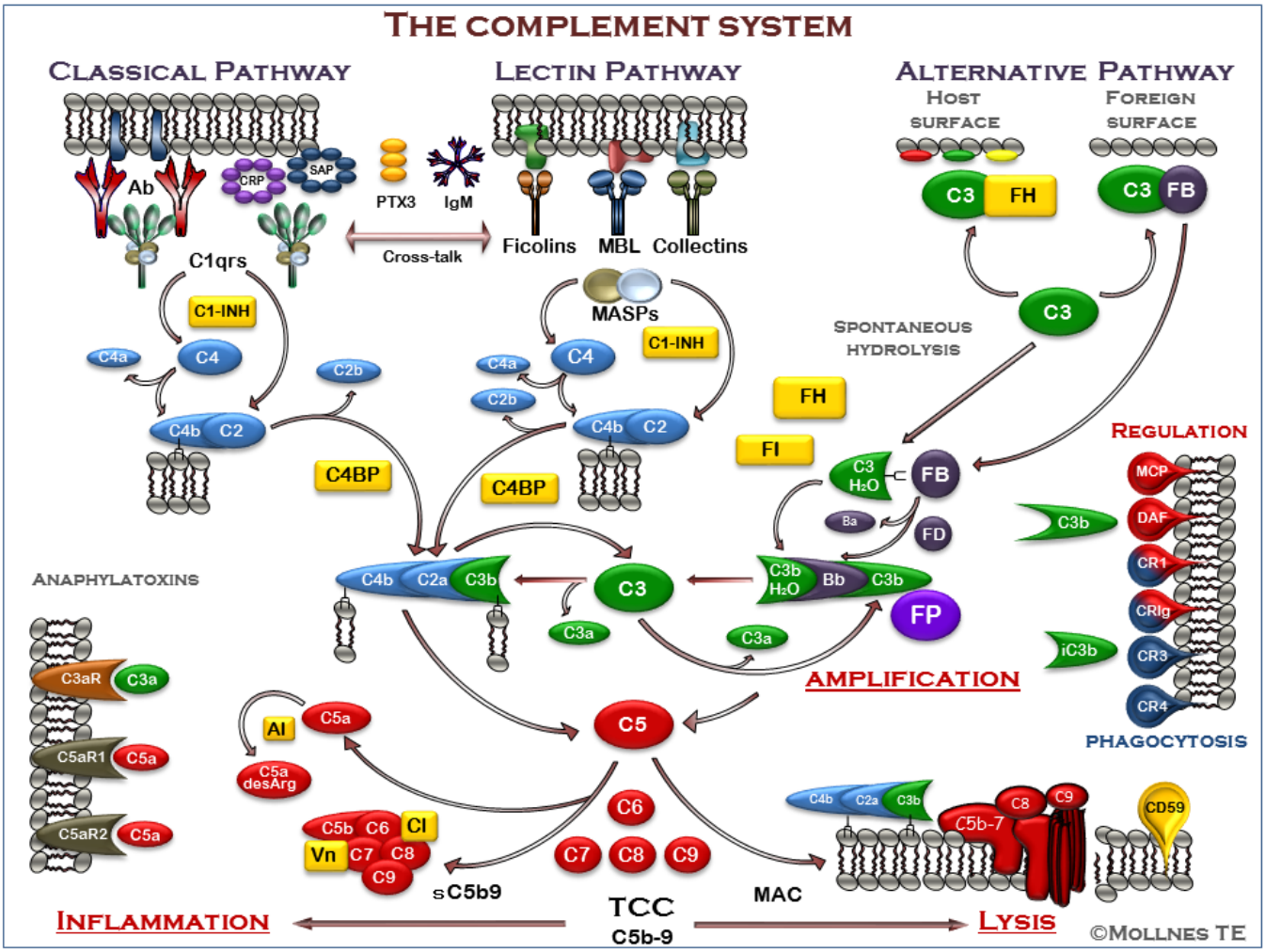

FIGURE 1. The Complement System

The complement system can be activated through three pathways (upper part of figure), all converging at the cleavage of C3 to generate C3a and C3b (middle part of figure).

The classical pathway is typically activated by antibodies, but also by pentraxins including C-reactive protein(CRP), serum amyloid P component (SAP), and pentraxin 3 (PTX3) can activate C1q.

The lectin pathway is activated through recognition of carbohydrates by mannose binding lectin (MBL), ficolins, and collectins. Furthermore, Lectin pathway activation can be mediated through IgM antibodies, e.g. it can be directed against damaged self-antigens.

The alternative pathway is activated by damaged cells or by foreign cells and is facilitated by the continuous spontaneous hydrolysis of C3. The alternative pathway also has an important function in the complement system by providing an amplification loop that enhances C3 activation independently of which pathway is initially activated. This effect is mainly due to properdin (FP), the only positive regulator in the complement system, which stabilizes the C3 convertase.

Activation of C3 leads to formation of a C5 convertase, which cleaves C5 into C5a and C5b. The anaphylatoxins C3a and C5a bind to the receptors C3aR, C5aR1, and C5aR2 leading to downstream production of inflammatory mediators (lower left part of figure). C5b initiates the formation of the terminal C5b-9 complement complex (TCC), which forms the membrane attack complex if inserted into a membrane (bottom part of figure) and leads to the lysis of bacteria and cells. In sublytic doses, the TCC activates inflammatory cells.

The cleavage and inactivation of C3b generates iC3b, which binds to complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) thus facilitating phagocytosis, oxidative burst, and downstream inflammation (right part of figure).

The complement system is tightly regulated by soluble inhibitors (marked in yellow), including C1 inhibitor (C1-INH), factor H (FH), factor I (FI), C4-binding protein (C4BP), anaphylatoxin inhibitor (AI), vitronectin (Vn), and clusterin (Cl), all of which keep the continuous low-grade activation in the fluid phase in check.

Host cell membranes are equipped with a number of inhibitors to protect them against attack by the complement system (right part of figure), including membrane cofactor protein (MCP;CD46), complement receptor 1 (CR1;CD35), decay accelerating factor (DAF;CD55), which controls C4 and C3 activation, and CD59, which protects against the final assembly of the C5b-9 complex..

1.2 BIOLOGICAL FUNCTION

Complement is a highly potent biological system that is intended to act locally to protect the host against danger. External dangers like pathogenic microbes express pathogen-associated molecular patterns (PAMPs) that are recognized by the host's pattern recognition molecules/receptors (PRMs/PRRs). The initial complement components are typically PRMs that react with the pathogen and initiate the subsequent response to fight infection. Complement deficiencies are thus associated with increased risk of infection.

The main mechanism of complement in infection defense is opsonization of pathogens by C4 and C3, leading to direct phagocytosis, mainly through CR3, or transport of the pathogens to reticular cells via binding to CR1. Rarely, the bacteria are lysed by complement. The C5b-9 complex can penetrate only a subgroup of Gram-negative bacteria, most importantly the Neisseria species. Thus, deficiency of the terminal C5-C9 components rarely leads to infectious diseases, but when these patients do suffer infections they are Neisseria infections. Interestingly, these patients have recurrent low-grade systemic infection, in contrast to complement-sufficient individuals, who often suffer from serious meningitis that can proceed to sepsis. Additionally, an important complement defense mechanism is the link between the complement system and adaptive immunity in which C3d bound on the surface of a pathogen binds to CD21 on the surface of B-cells and enhances antibody production by several orders of magnitude.

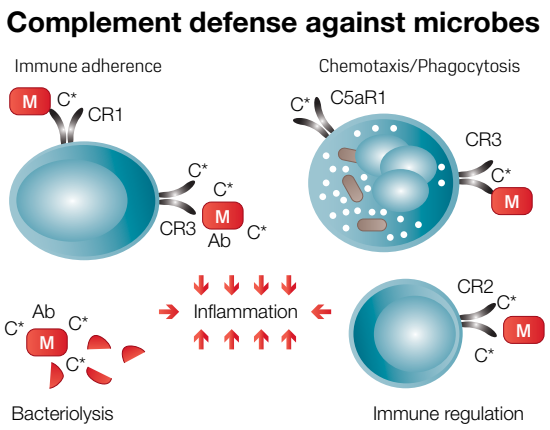

FIGURE 2. Complement in microbial defense.

Schematic illustration of four main mechanisms by which complement protects the host against microbes invading a tissue and leads to inflammation. For details, see the text.

C indicates an activated complement protein or fragment (e.g. C3b, C4b, C5a). M = microbe. Ab = antibody. CR = complement receptor.*

The mechanisms described above generally contribute to an inflammatory reaction in the tissue, which is a hallmark of complement activation. It is, however, of utmost important for the host to restrict the activation of the complement system, and the defense mechanisms should act locally. As soon as an infection or other activation of the complement cascade spreads and induces a systemic activation, the host will be more threatened by self-destructive uncontrolled complement activation than by the microbe itself. This is similar to the avoidance of uncontrolled activation of the coagulation system to avoid disseminated intravascular coagulation.

In addition to recognizing exogenous PAMPs to fight infection, the complement PRM also senses endogenous danger from damage-associated molecular patterns (DAMPs) that are exposed upon tissue damage from events such as ischemia reperfusion (e.g. myocardial infarction, stroke, and transplantation), trauma, and other sterile inflammatory conditions. Molecules that are normally hidden (frequently called alarmins) and molecules that change their structure (neoepitopes), become exposed to the complement components upon tissue damage. Such structures can be recognized by naturally occurring IgM antibodies that activate the classical pathway, by the lectin pathway PRM, or by the alternative pathway when discriminating an intact self-surface from a damaged or foreign surface. The sterile inflammation occurring due to damaged self tissue might induce tissue damage and organ dysfunction, and in case of systemic activation, such as multiple trauma, a systemic inflammatory response might occur leading to multi-organ failure and death.

The complement system does not always induce inflammation. It has an opposite role when it comes to embryotic organ development, especially with regard to synapse formation during nervous system development. Furthermore, it contributes to tissue renovation, typically illustrated by development of autoimmune systemic lupus erythematosus (SLE)-like conditions when C1q or other early components are genetically missing. A number of experimental studies indicate that an intact complement system is required for tissue regeneration and homeostasis.

The complement system is thus a double-edged sword playing friend and foe depending on the actual circumstances. Most importantly, it does not play alone, but is part of an orchestra cross-talking with a number of other biological systems, including the other plasma cascades and the other branches of innate immunity, including toll-like receptors, as well as with metabolic and neuroendocrine systems.

2. DEFICIENCY & DYSREGULATION: CLINICAL MANIFESTATIONS

2.1 PATHOPHYSIOLOGY DUE TO DEFICIENCIES

The traditional complement deficiencies of ordinary components (not the regulators) imply a reduced capacity of complement to be activated when necessary. This mainly implies increased risk of infections or a reduced capacity to renovate tissue debris leading to autoimmune-like diseases.

INFECTIONS

Recurrent infections are frequently seen in patients with complement deficiencies. However, taking into account that complement deficiencies are rare (except for MBL), patients with infections would only rarely present with a complement defect. In certain cases it is still important to include complement tests, in particular in patients with recurrent bacterial infections early in life and where other immune deficiencies cannot explain the condition.

Furthermore, *Neisseria* infections are associated with complement deficiencies in the terminal pathway (C5-C9), in particular if there is a family history, if the strain is atypical, or if the infection is systemic and recurrent. The frequency of meningococcal disease reported in C5-C9–deficient individuals reflects a 1,000 to 10,000-fold higher risk in this population. There is also a high prevalence of properdin deficiency in patients with meningococcal disease. The most common type 1 deficiency is characterized by the absence of properdin in plasma, whereas in type 2 deficiency properdin is low but detectable (<10% of normal). Notably, properdin defects are not detected in conventional hemolytic assays (AH50), but a novel ELISA-based total complement activity test (Wieslab®) shows low alternative pathway activity in sera with properdin deficiency (see below).

Many individuals with complement deficiencies, e.g. deficiencies of C2, have no disease. In fact, the first two C2 deficiencies were discovered in the early 1960s independently by two immunologists who could not get their own serum samples to work in the classical hemolytic assay, they were healthy otherwise. With this being said, more than half of those with C2 deficiency suffer recurrent infections and require careful follow-up, including vaccinations. Other early-stage deficiencies like C3, however, lead to serious infections early in life. It is likely that many complement defects are never diagnosed prior to the death of the individual due to severe infections. Therefore, it is critically important to include screening for complement deficiencies, especially if other immune defects are excluded.

MANNOSE BINDING LECTIN (MBL)

MBL is a key protein of the lectin pathway. MBL variant genes are found in 20%–30% of all individuals, and a complete deficiency in MBL is seen in approximately 5%. Most individuals with MBL deficiency are completely healthy, a fact emphasizing the broad redundancy of the innate host defense against infection. However, variant MBL genes are to some extent associated with increased infection risk. Data indicate that MBL is particularly important in early childhood between the ages of 6 and 18 months, pointing at the important role of innate immunity before the establishment and development of the infant's own adaptive immune system. Furthermore, immunocompromised patients, due to hematological disease or immunosuppressive treatment, also suffer infections. Thus, it is recommendable to add MBL to the list of parameters to be tested for when immunodeficiency is suspected, although interpretation of such results should be done with caution.

Leukocyte adhesion deficiency type 1 is caused by CD18 deficiency with impaired CR3 and CR4 function. This leads to severe infections and is diagnosed with flow cytometry.

AUTOIMMUNE DISEASES

Complement defects, particularly of the classical pathway, are frequently associated with SLE-like autoimmune disease. The strength of the association of a complement deficiency with SLE increases from C2 (10% prevalence) through C1r/s (57% prevalence) and C4 (75% prevalence) to C1q (95% prevalence). However, it should be emphasized that although complement deficiencies are frequently associated with autoimmunity, autoimmunity is only occasionally associated with complement deficiency. In active SLE, particularly with renal involvement, low total complement activity and low C4 are more often due to enhanced *in vivo* activation and thus to an acquired deficiency rather than to a genetic deficiency.

Neither total complement activity tests nor complement activation products are currently indicated as routine tests in patients with autoimmune conditions, but as research tools the activation products are highly relevant. In contrast, fluctuation in C4 levels is a reasonably good indicator of disease activity and is used routinely. Only occasionally is it indicated to measure total complement activity in autoimmune diseases, and in such cases only if C4 is normal. The reason for this is that a low C4 level due to consumption will give low levels of complement activity through the classical and lectin pathways. If C4 is normal and there is a family history of complement abnormalities, screening of complement activity or direct C1q measurement should be performed. A continuously undetectable level C4 in autoimmune diseases occasionally indicates a C4 deficiency, and it might thus be indicated to proceed with genetic analysis.

2.2. PATHOPHYSIOLOGY DUE TO DYSREGULATION

In recent years, the focus on the complement system in pathophysiology and disease has changed from the traditional ordinary complement deficiencies that lead to infections and autoimmunity to complement dysregulation due to genetic defects and mutations in the regulatory proteins, e.g. "loss of function" of factor H or mutations in the ordinary components, e.g. C3, leading to "gain of function" – both giving similar phenotypes and leading to diseases due to enhanced and detrimental complement activation. There are most likely a large number of diseases covered under this umbrella, and such diseases suggest that complement inhibition might be a viable therapeutic option. A limited number of these diseases are described below.

C1-INHIBITOR (C1-INH) & HEREDITARY ANGIOEDEMA (HAE)

HAE is an autosomal dominant condition with reduced concentration (type 1) or function (type 2) of C1-INH. Considering the life-threatening consequences of edema formation, early diagnosis of the C1-inhibitor deficiency in these patients is extremely important. The pathophysiology of HAE is complex, but it is now generally accepted that formation of bradykinin through activation of the kallikrein-kinin system, which is also controlled by C1-INH, is the major inductor of the edema. Thus, HAE might not be regarded among the complement dysfunction diseases, but because the diagnosis is based on complement analysis, it has a natural place on this list.

The diagnosis is based on C1-INH and C4 quantification. It is important to include both antigenic and functional assays for C1-INH because 15% of the patients have type 2 HAE with normal or even increased antigen concentrations of C1-INH. C4 is usually low in both types, even between the attacks, and serves as a valuable supplement to C1-INH analysis. An acquired condition (type 3) with a clinical picture identical to HAE might occur in patients who develop autoantibodies against C1-INH, which is frequently related to hematologic malignancies. In contrast to the hereditary forms, the acquired form has low concentrations of C1q. Mutations in Factor XII and ANGPT1 show a similar phenotype as C1-INH deficiency.

Treatment of HAE attacks has typically been the administration of high concentrations of C1-INH, but because it is now clear that bradykinin is the mediator of the clinical effects, treatment has gradually shifted to bradykinin receptor antagonists. Although C1-INH is not a specific complement inhibitor, it might still have usefulness in treating conditions where several of the plasma cascades are activated. If any effects are observed, the target(s) should be interpreted with caution because it might well be that inhibition of the kallikrein-kinin system or the coagulation/fibrinolytic systems is behind the effect more so than the complement system.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

A somatic mutation in a bone marrow cell gene coding for the phosphatidylinositol anchor (PIG-A) results in the clonal development of cells with decreased expression of membrane proteins linked to PIG-A, including the complement regulators CD55 and CD59. This renders red blood cells susceptible to complement-mediated lysis, which is the hallmark of this condition. Diagnosis of PNH was traditionally made using Ham's test (an acid lysis test), but it is now specifically assessed by flow cytometric analysis of the respective cell surface proteins. The cell lysis that occurs is mainly intravascular and completely complement dependent. The patients suffer from anemia and thromboses, and treatment has traditionally been blood transfusions. PNH was the first disease approved by the FDA for treatment with the complement inhibitor eculizumab (Soliris®), a monoclonal antibody blocking cleavage of C5. The treatment is effective but expensive. Most of the patients do not need transfusions and do not suffer from thromboses when treated with this drug. Complement tests are useful for monitoring treated patients.

ATYPICAL HEMOLYTIC UREMIC SYNDROME (AHUS)

aHUS, one of the thrombotic microangiopathies, is a severe disease often occurring in children and in many cases leads to kidney failure and the need for transplantation. Approximately two thirds of the patients have dysregulation of the complement system

with increased activation due to loss of function mutations in a regulatory protein (e.g. factor H, factor I, or membrane cofactor protein (CD46)) or to gain of function mutations in the ordinary components (e.g. C3 or factor B). It is important to note that the concentration of the actual component in serum might be normal and it might be the function that is impaired. aHUS is also FDA approved for treatment with eculizumab, and the treatment is generally highly effective. In contrast to PNH, which needs life-long treatment, many aHUS patients remain in remission after discontinuing the treatment. They are closely followed, and in case of exacerbation treatment is re-started.

The presence of autoantibodies against factor H or against the C3, C4, or C5 convertases ("nephritic factors") gives a similar phenotype to patients with genetic defects, and thus it is important to test for these autoantibodies in order to decide on the correct treatment.

RENAL DISEASES

Dysregulation of the alternative pathway with particular involvement of C3 leads to diseases classified as C3 glomerulopathies (C3G). Among these is dense deposit disease, previously termed membranoproliferative glomerulonephritis type II, and if the main feature is glomerulonephritis the condition is termed C3GN. Many of these patients have low levels of serum C3 due to consumption and consequently display low activity in all pathways in the total complement activity test. In other renal diseases, the pathophysiology is often more complex. A role for complement has been suggested in IgA nephropathy, lupus nephritis, and other forms of glomerulonephritis, and the complement system is particularly important in antibody-mediated kidney transplant rejection. Case reports indicate that some of these conditions might benefit from complement inhibitory treatment, but controlled trials are still lacking.

NEUROLOGICAL AND EYE DISEASES

A role for complement in the pathogenesis of several neurodegenerative diseases has been suggested, including Guillain-Barre syndrome and neurodegenerative disorders such as Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis. Two diseases of particular interest are associated with autoantibodies, and activation occurs through the classical pathway. The first is myasthenia gravis in which patients present with autoantibodies to the acetylcholine receptor, which impair neuromuscular transmission signals. The second is neuromyelitis optica, which was previously regarded as a subgroup of multiple sclerosis with vision impairment but is now known to be a separate entity where the pathogenic factor is autoantibodies against aquaporin 4. These two diseases might benefit from complement inhibition, e.g. with a blocking antibody to C1s to halt activation at an early step, or with a C5 inhibitor because C5b-9 is probably crucial for the tissue damage that occurs in this disease. Clinical trials of complement inhibition are ongoing in myasthenia gravis patients.

Age-related macular degeneration (AMD) is the most common cause of blindness in industrialized countries. It is closely related to mutations in the same genes as described for aHUS above, indicating a dysregulation of the alternative pathway. Clinical trials blocking factor D are underway. There is also evidence for a role of the complement system in chorioretinitis and possibly also in glaucoma.

HOW MANY DISEASES?

Above is just a sample of some of the conditions for which human or animal studies support a role for the complement system and thus might be candidates for future therapy.

However, the list is much longer than this, including diseases like ANCA vasculitis, HELLP syndrome, catastrophic anti-phospholipid syndrome, cold-agglutination, lung diseases, allergies, atherosclerosis, ischemia-reperfusion injury like myocardial infarction and stroke, trauma, sepsis leading to systemic inflammatory response syndrome, and many more. Thus the question is: In how many diseases is the complement system NOT involved in the pathogenesis in some way or another?

3. COMPLEMENT TESTS

3.1. SCREENING FOR DEFICIENCIES

Screening of the functional activity of the complement system is first of all indicated when a deficiency of complement cannot be excluded as a cause of immune disturbances, primarily in recurrent infections and occasionally in autoimmune diseases. These tests have traditionally been performed using hemolytic assays, the principles of which are illustrated in Figure 3. These assays provide insight into the integrity of the entire cascade of reactions. For the classical pathway, serial dilutions of the sample to be analyzed are incubated with antibody-sensitized sheep erythrocytes. The results are usually expressed as a reciprocal dilution of the sample required to produce 50% lysis, which is known as the CH50 test.

Tests evaluating the functional activity of the alternative pathway (AH50) also work with lysis as the readout, but with non-treated rabbit erythrocytes (alternatively chicken or guinea pig erythrocytes) as target cells. Activation of the classical pathway has to be blocked by adding EGTA to chelate Ca2+ and by adding an optimal concentration of Mg2+.

A novel methodological approach, an enzyme-linked immunosorbent assay (ELISA)-based total complement screen (Wieslab®), has been developed that separately detects the complement activity of the classical, lectin, and alternative pathways (Figure 4).

The principle is thus similar to the hemolytic tests, but the readout is not dependent on red blood cell lysis. Instead, deposition of a monoclonal antibody reacting with a neoepitope in activated C9 is the readout.

Reduced activity will be seen if one or more components are low or missing. Properdin deficiencies are detected in the AP assay.

The activity of the three pathways is tested in microtiter wells coated with IgM, mannan, or lipopolysaccharide, respectively. When adding serum, the activation will continue until the assembly of C5b-C9, which can then be quantified by an antibody specific for activated C9 (figure 4). Thus, the principle is very similar to the hemolytic assays, but this assay is more robust because it is not dependent on the quality of living cells, and it is suitable for large-scale complement deficiency screening. The following defects should be considered depending on the pattern observed in the screening:

- Abolished CP, normal LP and AP: C1q, C1r, C1s.
- Abolished LP, normal CP and AP: MBL, MASPs.
- Abolished AP, normal CP and LP: properdin, factor D and B.
- Abolished CP and LP, normal AP: C2, C4.
- Abolished CP, LP, and AP: C3, C5, C6, C7, C8, C9.

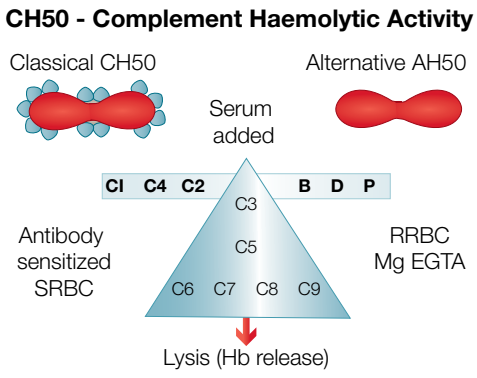

FIGURE 3. *Schematic illustration of hemolytic screening tests for the activity of the classical (CH50) and alternative (AH50) pathway. Serum is added to antibody sensitized sheep red blood cells (SRBC) for the CH50 test. If all components of the classical and terminal pathway are present, the red blood cells will lyse and the readout is released hemoglobin (Hb).*

Similarly, AH50 is measured using rabbit red blood cells (RRBC). Unfortunately, this assay does not detect properdin deficiency, which is of clinical importance.

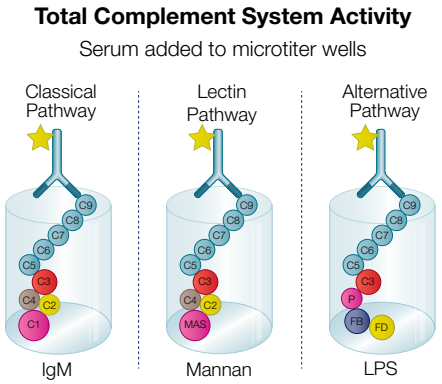

FIGURE 4. *Schematic illustration of the Wieslab® ELISA-based screening test for separate detection of classical pathway (CP), Lectin pathway (LP), and Alternative pathway (AP) activation. Serum is added to the wells and allowed to react with the specific activators. If all components are present in the serum, the cascade will continue until activated C9 is bound.*

3.2. INDIVIDUAL COMPONENTS

When defects are detected in the screening assay, specific assays for single components can be performed either by functional analysis, similar to the hemolytic assays described above using specific deficient sera in the test, or by immunochemical quantification. The latter can be performed using immunoprecipitation tests (radial immunodiffusion or nephelometer techniques), by ELISA, or by western blot. It is important to note that the guidelines for the handling of the samples are strictly different for functional analysis and immunochemical quantification.

From the initial screening, it is thus possible to select for candidate complement protein deficiency, which should then be verified by quantifying the individual components. Homozygous factor B deficiencies have to date been documented in only one case, and factor D in only a few families. Furthermore, lack of LP activity is in >99% of cases due to a defect in MBL because only a very few MASP defects have been documented.

Total complement activity is sensitive to in vitro activation of complement in serum. If serum is heat inactivated, has been stored for a long time at room temperature (see "treatment of samples" below), or contains complement-activating agents (e.g. immune complexes, cold agglutinins), the activity is reduced and may even be zero. Thus, if the activity of all pathways is abolished, a new sample with fresh serum should always be tested before further investigation is done.

If a defect is verified by protein quantification, further functional assays are not required. On the other hand, if immunochemical assays do not reveal any deficiency, the actual component might be functionally inactive, and a functional or genetic assay can then verify the diagnosis. In certain cases, direct identification of single components without total complement activity screening is indicated. Thus, in routine diagnosis, C3 and C4 are most frequently measured, particularly associated with autoimmune vasculitis and glomerulonephritis. Despite their long tradition of use, the results of such measurements should be interpreted with caution because several factors influence serum C3 and C4 concentrations. **1)** They are produced by the liver, thus liver failure will reduce their levels. **2)** They are acute-phase reactants and will increase during inflammation. **3)** They might be decreased due to in vivo activation and consumption. 4) Many patients are in an intensive care unit and are highly diluted, and thus all proteins will be lowered, including C3 and C4. Thus, it is the combination of these four factors that determines the serum level. An illustration of the differences seen in C3 and C4 measurements is given in Table 1

	Patient 1 F24)	Patient 2 (F35)	Reference range
C3	0.20	0.30	0.50 - 1.00 g/L
C4	0.09	0.06	0.10 - 0.50 g/L
C3dg	25	126	20 - 45 AU/mL
sC5b-9	3.9	15	2.2 - 6.6 AU/mL
Diagnosis:	Liver failure	Chronic active hepatitis	

TABLE 1.
Illustration of the advantage of supplementing with complement activation products in order to interpret C3 and C4 levels.
Two females aged 24 and 35 years presented with low C3 and C4 levels. One had liver failure with reduced synthesis of C3 and C4 and low activation products (C3dg, sC5b-9), while the other had hepatitis without liver failure, but with substantially increased activation products consistent with in vivo complement activation leading to C3 and C4 consumption. Thus, two completely different pathophysiological states led to similar C3 and C4 profiles.

3.3. ACTIVATION PRODUCTS

A low concentration of a complement component might be due to genetic deficiency, decreased synthesis (liver failure), or increased consumption due to in vivo activation, whereas acute-phase reactions might increase the concentrations of a number of the components. Thus, the level of a single component might be difficult to interpret. In order to evaluate the degree of activation of complement in vivo, specific activation products need to be quantified. Many novel sensitive, specific, and reliable enzyme immunoassays to detect complement activation products are based on monoclonal antibodies directed against neoepitopes, i.e. antigenic determinants exposed in the activation products but hidden in the native components. When an anti-neoepitope–specific antibody is used as the capture antibody in the assay, only the actual activation product will bind. After washing, there are no native components left in the well that can compete with the activation product, and a second antibody, not necessarily neoepitope specific, can then be used for detection. With this approach the exact amount of an activation product can be quantified directly in a plasma sample (Figure 5).

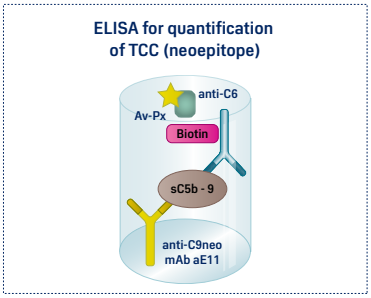

FIGURE 5. *Detection of complement activation products.*

Schematic illustration of using an ELISA to quantify an activation product. The soluble TCC, sC5b-9, is measured in plasma by a capture monoclonal antibody reacting with a neoepitope in activated C9 (aE11) and detected with an anti-C6 antibody.

The principle is the same as for the screening assay – both are detecting the TCC – but the screening assay detects the in vitro formation of C5b-9 on a surface whereas the activation-product ELISA detects soluble sC5b-9.

A number of assays have been designed to detect complement activation products from different parts of the cascade. sC5b-9 indicates that the terminal pathway has been activated through to its end, irrespective of which initial pathway was activated, and sC5b-9 is less prone to in vitro activation compared to most of the other activation products. Together with a C3 activation product (e.g. C3a, C3bc, C3dg), sC5b-9 serves a good general "screening" for activation of the complement system. However, in order to evaluate which of the initial pathway(s) are activated, other assays have to be used. C4 activation products (e.g. C4a, C4bc, C4d) indicate classical and/or lectin pathway activation, and C1rs-C1INH complexes reflect classical pathway activation. Alternative pathway activation is reflected by factor B activation (Ba, Bb) or convertase formation (C3bBbP). Terminal pathway activation can be detected by C5a in addition to sC5b-9. Specific assays for activation products of the lectin pathway in the fluid phase have not yet been designed.

Complement activation products are usually present only in trace amounts in vivo, but they are rapidly generated in vitro. Therefore, it is crucial that the samples are collected and stored properly in order to avoid in vitro activation.

The early components C3 and in particular C4 are highly prone to in vitro activation, which should be kept in mind if increased concentrations of their activation products are detected in samples that are not stored properly. Furthermore, the various complement activation products have different half-lives in vivo. This is important for the activation product(s) of choice to be measured and for the interpretation of the data. Due to rapid receptor binding, the biologically highly active and important C5a fragment has a half-life of approximately one minute and is thus difficult to detect in samples obtained in vivo, whereas the various C3 activation products are readily detectable due to half-lives of a few hours. sC5b-9 has a half-life of 50–60 minutes. Thawing and freezing also increases many of the activation products, in particular those in the early part of the cascade, whereas sC5b-9 is rather stable.

If measuring an activation product from a single component, the amount is best related to the concentration of the native component because a low level of native component would yield smaller amounts of activation products during in vivo activation. Thus, it has been postulated that the ratio between the activation product and the native component is a more sensitive indicator of in vivo activation than the activation product alone, e.g. the ratio C3dg/C3 will be relatively higher if C3 activation is strong leading to C3 consumption and low C3 levels. Routine use of activation products is still limited, partly due to the strict guidelines for sample handling and partly due to lack of clinical experience. Experimentally, however, these tests are widely used to study complement activation and the effects of complement inhibition. Because complement inhibition therapy is now increasing clinically, it is likely that the need for these tests will also increase. In particular, testing for sC5b-9 will be informative when a C5 inhibitor is used (see 3.5).

3.4. DYSREGULATION AND GENETICS

In the new era with complement therapeutics reaching the clinic, the focus is to a great extent on disease conditions due to dysregulation, especially those due to defects in the regulators.

In the case of PNH, diagnosis is rapidly done using flow cytometry. If the clinical features suggest aHUS or a form of C3GN, rapid immunological analysis of autoantibodies can be performed (anti-factor H, nephritic factors). Except for these analyses, few complement tests can be of immediate help in the diagnosis. C3, C4, a total complement screen test, and an activation product test might be valuable as a supplementary test, but these might be normal despite a functional defect in, for example, factor H. If the mutation involves binding to endothelial cells in glomeruli, it will lead to complement attack on these cells because factor H acts as an important surface bound molecule on endothelial cells that protects against self-attack, but this might not affect any of the serum and plasma complement analyses mentioned above. These are, therefore, not diagnostic, and a genetic test is required. A problem with the genetic tests has been the long response time, but with the new generation of genetic analysis methods it should be possible to get the results after a few days. "Disease packages" have been set up by many laboratories, implying that when the clinical diagnosis is, for example, aHUS, the laboratory performs genetics analysis for the proteins known to be associated with the disease (e.g. Factor H, Factor I, MCP, C3, factor B). The importance of such tests will increase in the future, both because new mutations are continuously being found in the diseases already known to be related to complement, and because new diseases will certainly be added to the list of complement dysfunctions.

3.5. COMPLEMENT IN THE THERAPEUTIC ERA

Treatment of patients with a complement inhibitor challenges the complement laboratory field to develop and validate methods to monitor the efficacy of the drug. The only drug targeting the complement system in clinical use today is eculizumab, which inhibits the activation of C5. Other complement inhibitors are in clinical trials, and potential new drugs are continuously being developed. Specific inhibition of the initial pathways might be obtained, for example, by targeting C1s (the classical pathway), MASP-2 (the lectin pathway), or factor D or factor B (the alternative pathway). C3 is a potent component to target because it is the first common molecule for all three initial activation pathways. C3aR might also be a possible target in certain conditions. Other C5 inhibitors are also in clinical trials, including agents that block C5 cleavage, like eculizumab, or block C5a or its receptors C5aR1/2, with the latter approaches leaving the C5b-9 pathway open to form the complex.

Complement tests will be increasingly important because complement inhibition is expanding in the clinic and novel inhibitors are being developed. Many tests are already available as described above, and these will be useful for testing the efficacy of potential inhibitors both in vitro and in vivo. The functional activity measurement of C5b-9 deposition, as measured in the Wieslab® assay, will be particularly useful because it covers the whole complement cascade. Thus, blocking the function of any of the ordinary native components can be evaluated using this test, with the added advantage of the test being standardized, rapid, and easy to perform. Inhibitors blocking complement receptors (e.g. C5aR1) and/or activation products (e.g. C5a) will, however, require the development of novel assays for testing their efficacy.

Future pharmacological treatment will in general focus on individualized therapy, i.e. giving optimal doses to obtain effective treatment, avoiding adverse effects, and reducing cost. This will also be a challenge for complement inhibition therapy. Thus, it will be important to design and validate complement assays that can guide clinicians in follow-up of these patients to ensure that they are receiving the right dose at the right time.

4. TREATMENT OF SAMPLES

Correct treatment of samples to be analyzed for complement is critically important in order to obtain reliable results. The following guidelines are recommended:

1. **Total complement activity** (screening with hemolytic assays or the Wieslab® total complement screen): Fresh normal serum prepared from whole blood without anticoagulants and allowed to clot for 1–2 hours. Storage at 4°C until frozen at –70°C, preferentially within the same day (6–8 hours). If transport to a hospital is needed, 24 hours before freezing is acceptable, and if the result is normal, the sample is fully reliable (see "To be noted" below).

2. **Functional assays of single proteins** (e.g. C1-INH function): Fresh normal serum as described above for the total complement activity.

3. **Protein quantification of single components** (e.g. C1-INH, C1q, C3, C4, MBL) **and autoantibodies** (e.g. anti-factor H, nephritic factors): Normal serum. If possible, handling should be as described above under item 1 because it might be necessary to also use these samples for functional analysis. However, if only the protein concentration is to be measured, the length of storage is not critical because the assays used are normally not sensitive to in vitro activation.

4. **Complement activation products.** Sampling and handling is critically important to avoid activation in vitro. Blood must be drawn directly into EDTA-containing tubes, turned gently 4 or 5 times, and placed on slushed ice until centrifuged (preferentially in a cooling centrifuge) for 15 min at 1,500 x g. Plasma should be removed carefully to avoid cellular elements and stored immediately at –70°C.

5. **Genetic testing.** Fresh or frozen EDTA whole blood sent on dry ice.

TO BE NOTED:

* You can trust a normal complement result (unless the sample has been mixed and is not from the actual patient). A pathological in vivo complement condition cannot change into a normal in vitro condition, but the opposite can occur.

* If a pathological result is obtained for a functional test (including total complement screening) or for an activation product, the test should be repeated with a new sample documented to have been obtained and stored according to the guidelines before proceeding with more extensive analyses.

WIESLAB® KITS FOR ASSESSMENT OF COMPLEMENT ACTIVITY

INTENDED USE

The Wieslab® Complement system kit is a set of enzyme immunoassays for the qualitative determination of functional activity of classical, Lectin (MBL), and alternative complement pathways in human serum. Four different kits are available, one for each pathway and a combined screening kit for all three pathways.

PRINCIPLE OF THE WIESLAB® COMPLEMENT ASSAY

The Wieslab® Complement assay combines principles of the hemolytic assay for complement activation with the use of labeled antibodies specific for a neoantigen produced as a result of complement activation. The amount of neoantigen generated is proportional to the functional activity of the complement pathways. The wells of the microtiter strips are coated with specific activators of the classical, MBL, or alternative pathway. Patient serum is diluted in diluent containing specific blockers to ensure that only the respective pathway is activated.

During the incubation of the diluted patient serum in the wells, complement is activated by the specific coating. The wells are then washed, and C5b-9 is detected with a specific alkaline phosphatase-labeled antibody specific to the neoantigen expressed during MAC formation. After a further washing step, detection of specific antibodies is obtained by incubation with alkaline phosphatase substrate solution. The amount of complement activation correlates with the color intensity and is measured in terms of absorbance (optical density (OD)).

PERFORMANCE CHARACTERISTICS

Sera samples from 120 blood donors were tested in the three assays, and the normal reference range was calculated. The values were expressed as a percentage of the positive control (Table 2). In the Classical pathway assay, no blood donor was below 40%. In the Lectin (MBL) pathway assay, 23 samples were below 10%, and they had MBL values (established in a separate assay) below 500 ng/ml. In the Alternative pathway assay, no blood donor was below 10%.

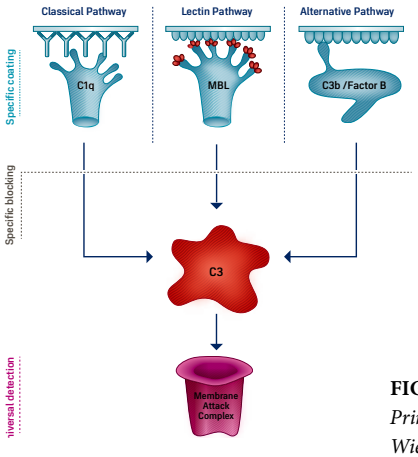

FIGURE 1.
Principle of the Wieslab® Complement ELISA system

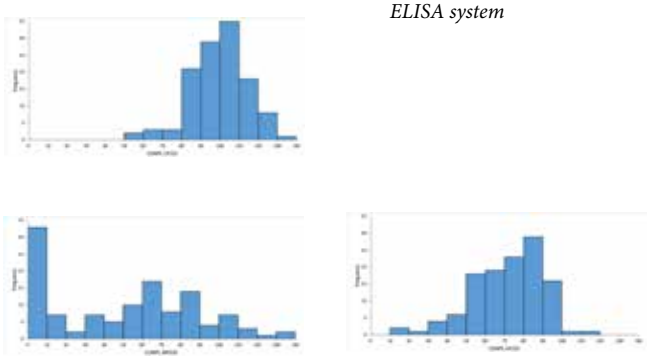

TABLE 2.

	n	Mean (%)	±2SD (%)	Median
Classical pathway	120	99	69-129	100
MBL pathway	120	49	0-125	56
Alternative pathway	120	71	30-113	73

TABLE 3.
Sera with known complement deficiencies were tested in the assays and the following results were obtained. All deficient sera were detected in the assay and gave values below 5%.

Deficiency	C1q	C2	C3	C4	C5	C6	C7	C8	C9	P	H	I
Number of patients	3	11	1	1	2	1	2	2	1	9	1	2
Number of deficient sera detected	3	11	1	1	2	1	2	2	1	9	1	2

COMPL 300	ELISA kit for total functional assessment of the complement system (96 wells breakapart)
COMPL CP310	ELISA kit for total functional assessment of the Classical pathway (96 wells breakapart)
COMPL MP320	ELISA kit for total functional assessment of the Lectin (MBL) pathway (96 wells breakapart)
COMPL AP330	ELISA kit for total functional assessment of the Alternative pathway (96 wells breakapart)

Answers in Life Science

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