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2. Complement System

Dr. Maitreyee Mondal

Assistant Professor,
Department of Microbiology,
Dinabandhu Andrews College, Baishnabghata,
Garia, Kolkata, India.

2.1 Introduction:

The complement system is an integral part of the innate immune response and acts as a bridge between innate and acquired immunity. This system is composed of a series of proteins that are mostly (although not exclusively) synthesised in the liver, and exist in the plasma and on cell surfaces as inactive precursors (zymogens). Complement mediates responses to inflammatory triggers through a co-ordinated sequential enzyme cascade which leads to clearance of foreign cells through pathogen recognition, opsonisation and lysis. Complement also possesses anti-inflammatory functions: it binds to immune complexes and apoptotic cells, and assists in their removal from the circulation and damaged tissues. The complement proteins are activated by, and work with IgG and IgM antibodies, hence the name 'complement'. Many complement proteins exist in a 'precursor' form and are activated at the site of inflammation. The complement system is more complex than many enzymatic cascades as it requires the formation of sequential non-covalently associated activated protein fragments. These in turn become convertases and cleave components for the next enzymatic complex in the cascade, and the rapid dissociation of these complexes (and loss of enzymatic activity) forms an integral part of the elegant regulation of complement activity.

2.2 History:

In the late 19th century, the focus of scientific research was on the human body's defence against microbial infection. The 'Theory of Metchnikoff' proposed that phagocytes in the blood were capable of ingesting and destroying the invading bacteria, thus providing the basis of innate cellular immunity.

This phagocytic theory was challenged by many pathologists initially on the basis that the phagocytic leucocytes were 'truly causal in the successful response to infection'. Buchner and colleagues (1891) found a heat labile factor in blood that was capable of killing bacteria, and named it 'alexin' (in Greek, means 'to ward off').

Jules Bordet supported this 'humoral theory' (immunity conferred due to antitoxic and bactericidal substances in body fluids) by demonstrating that immune lysis required the presence of two factors: a heat labile lytic factor (similar to alexin) and a heat-stable factor, which he termed sensitiser (which we now know was antibody).

Paul Ehrlich described the side-chain theory of antibody formation, especially the mechanisms of antibody neutralisation by toxins that induced bacterial lysis with the help of complement (which has replaced the historical term alexin).

According to his theory, the immune cells contained receptors that could recognise antigens, and following immunisation, these receptors multiplied and were shed into the circulation as 'amboceptors' (now called antibodies).

These antibodies attached not only to specific antigens but also to a heat-labile antimicrobial component called 'complement'. Ehrlich's theory proposed that the antibody and complement combined to form a complex enzyme capable of attacking and killing cells and micro-organisms.

2.3 The Complement Components:

The proteins and glycoproteins that compose the complement system are synthesized mainly by liver hepatocytes, although significant amounts are also produced by blood monocytes, tissue macrophages, and epithelial cells of the gastrointestinal and genitourinary tracts.

These components constitute 5% (by weight) of the serum globulin fraction. Most circulate in the serum in functionally inactive forms as proenzymes, or zymogens, which are inactive until proteolytic cleavage, which removes an inhibitory fragment and exposes the active site. The complement-reaction sequence starts with an enzyme cascade.

Complement components are designated by numerals (C1–C9), by letter symbols (e.g., factor D), or by trivial names (e.g., homologous restriction factor). Peptide fragments formed by activation of a component are denoted by small letters. In most cases, the smaller fragment resulting from cleavage of a component is designated "a" and the larger fragment designated "b" (e.g., C3a, C3b; note that C2 is an exception: C2a is the larger cleavage fragment).

The larger fragments bind to the target near the site of activation, and the smaller fragments diffuse from the site and can initiate localized inflammatory responses by binding to specific receptors. The complement fragments interact with one another to form functional complexes. Those complexes that have enzymatic activity are designated by a bar over the number or symbol (e.g., C4b2a, C3bBb).

2.4 Pathways of Activation:

There are three known pathways for complement activation: Classical, Alternative and Lectin pathway (Fig. 1). The final steps that lead to a membrane attack are the same in all pathways.

2.4.1 The Classical Pathway Begins With Antigen-Antibody Binding:

The classical pathway is initiated by IgM or IgG antigen/ antibody complexes binding to C1q (first protein of the cascade) leading to activation of C1r, which in turn cleaves C1s. This in turn activates the serine proteases that lead to cleaving of C4 and C2, leading to formation of C4b2a (C3 convertase), which in turn cleaves C3 into C3a and C3b. While C3a acts as a recruiter of inflammatory cells (anaphylatoxin), C3b binds to the C4b2a complex to form C5 convertase (C4b2a3b).

The C5 convertase initiates the formation of the Membrane Attack Complex (MAC) that inserts into membrane creating functional pores in bacterial membranes leading to its lysis. The classical pathway can also be activated by other danger signals like C-reactive protein, viral proteins, poly-anions, apoptotic cells and amyloid, thus providing evidence that classical pathway could be activated independent of antibodies

2.4.2 The Alternative Pathway Is Antibody-Independent:

Fifty years after the discovery of the classical activation pathway, Pillemer et al. proposed a highly controversial alternative activation pathway. Initially, this was rejected by the scientific community and only substantiated and accepted more than a decade later. Pillemer's hypothesis was based on observations that the complement system could be activated by direct binding of bacteria and yeast independent of antibody interaction.

It was originally named the 'properdin pathway' and is now known as the alternative pathway. The alternative pathway is not so much an activation pathway, as it is a failure to regulate the low level continuous formation of a soluble C3 convertase.

The internal thioester bond of C3 is highly reactive and undergoes spontaneous hydrolysis resulting in a molecule known as C3 (H_2O) which resembles C3b. This can then bind to factor B, and be processed into a short lived soluble C3 convertase that can generate more C3b.

If this C3b binds to a nearby surface that is incapable of inactivating it (such as bacteria/yeast cells or damaged host tissues), this then leads to amplification of the alternative pathway.

The presence of complement regulators in healthy cells ensures the spontaneous hydrolysis of C3 is kept in check. C3 activation takes place when C3b binds to factor B and is then cleaved by factor D (a process which is stabilised by magnesium ions and properdin).

The enzymatic action of factor D acts as the rate limiting step of the alternative pathway and cleaves factor B, the larger fragment of which remains bound to C3b to form the alternative pathway C3 convertase–C3bBb. C3b is able to create new C3 convertase in the presence of Factors B and D, thus acting as an 'amplification loop' for other pathways, as well as the alternative pathway.

The C3bBb generated in the alternative pathway can activate unhydrolyzed C3 to generate more C3b autocatalytically. As a result, the initial steps are repeated and amplified, so that more than $2 \text{ X } 10^6$ molecules of C3b can be deposited on an

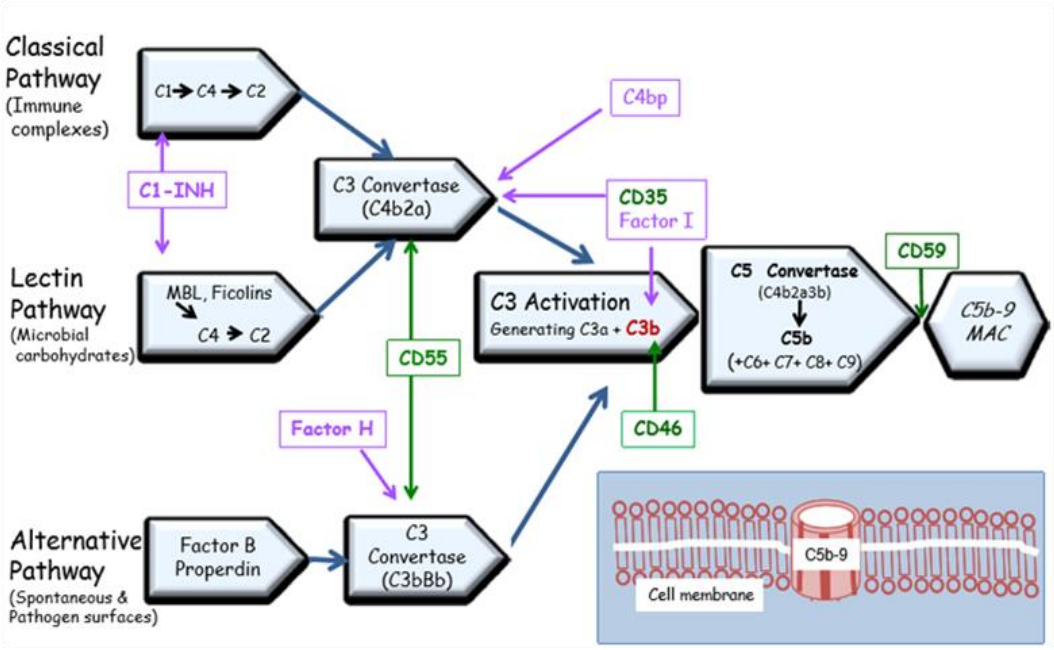


Figure 2.1: Pathways of complement activation: classical, alternative and lectin pathway: IgM or IgG antigen/antibody complexes binding to C1q, the first protein of the cascade, initiates the classical pathway. The alternative pathway is not so much an activation pathway, as it is a failure to regulate the low level continuous formation of a soluble C3 convertase.

The third pathway is known as MBL (Mannosebinding lectin)/MASP (MBL associated Serine Protease) pathway. The initiating molecules for the MBL pathway are multimeric lectin complexes that bind to specific carbohydrate patterns uncommon in the host, leading to activation of the pathway through enzymatic activity of MASP.

The sites of action of the membrane bound complement regulators–CD35, CD46, CD55 & CD59 (green boxes) and the fluid phase regulators – C1-INH, Factor H, Factor I and C4bp (violet boxes) are represented with arrows.

Insert: Membrane Attack Complex (MAC). The interaction of C5b with C6, C7, C8 and C9 leads to formation of C5b–9 or Membrane Attack Complex (MAC), a multimolecular structure that inserts into the membrane creating a functional pore leading to cell lysis.

Antigenic surface in less than 5 minutes. The C3 convertase activity of C3bBb generates the C3bBb3b complex, which exhibits C5 convertase activity, analogous to the C4b2a3b complex in the classical pathway. The nonenzymatic C3b component binds C5, and the Bb component subsequently hydrolyzes the bound C5 to generate C5a and C5b; the latter binds to the antigenic surface. The alternative pathway omits the components C1, C2 and C4.

2.4.3 The Lectin Pathway Originates with Host Proteins Binding Microbial Surfaces:

Forty years after the proposal of the alternative pathway, the MBL (mannose-binding lectin)/MASP (MBL-associated serine protease) pathway was discovered. This pathway was characterised by using proteins isolated from rabbit liver and serum, but its function remained unclear initially. The initiating molecules for this pathway are collectins (MBL and ficolin), which are multimeric lectin complexes. These bind to specific carbohydrate patterns uncommon in the host, leading to activation of the pathway through enzymatic activity of MASP. There are structural similarities shared between MBL and C1 complexes (MBL- with C1q-associated serine proteases, MASP-1 and MASP-2 with C1r and C1s, respectively), leading to the belief that complement activation by MBL and C1 complexes are similar. MASP-2 cleaves C4 and C2 to form C3 convertase, while MASP-1 may cleave C3 directly bypassing the C4b2a complex, albeit at a very slow rate. Another serine protease, MASP-3 was shown to down-regulate the C4 and C2 cleaving activity of MASP-2. Following the initial characterisation of MBL, 3 other lectins (known as ficolins) have been shown to interact with MASP: ficolin-1 (or M-ficolin), ficolin-2 (or L-ficolin) and ficolin-3 (or H-ficolin or Hakata antigen). The ficolins activate the lectin pathway by forming active complexes with MASP [51, 52]. More recently, a new C-type lectin (CL-11) was shown to interact with MASP-1 and/or MASP-3 and could activate the lectin pathway.

2.4.4 Other Activators of The Complement System:

Various serine proteases belonging to the coagulation system have also been shown to activate the complement cascade independent of the established pathways. In vitro findings suggested that the coagulation factors FXa, FXIa and plasmin can cleave both C5 and C3, leading to generation of anaphylatoxins C5a and C3a [54].

Studies have documented FVIII and von Willebrand factor to possess lectin activity. Vice versa, complement factors are also known to interact with the coagulation system. C1 inhibitor was shown to block the endogenous coagulation pathway, while C5a was shown to induce tissue factor (membrane glycoprotein that serves as a cofactor for blood coagulation factor VIIa) activity on endothelial cells.

Individual cells have also been implicated in activating certain elements of complement pathway. It has been showed that phagocytic cells, especially lung macrophages could generate C5a from C5 independent of the plasma complement system using cell bound serine proteases.

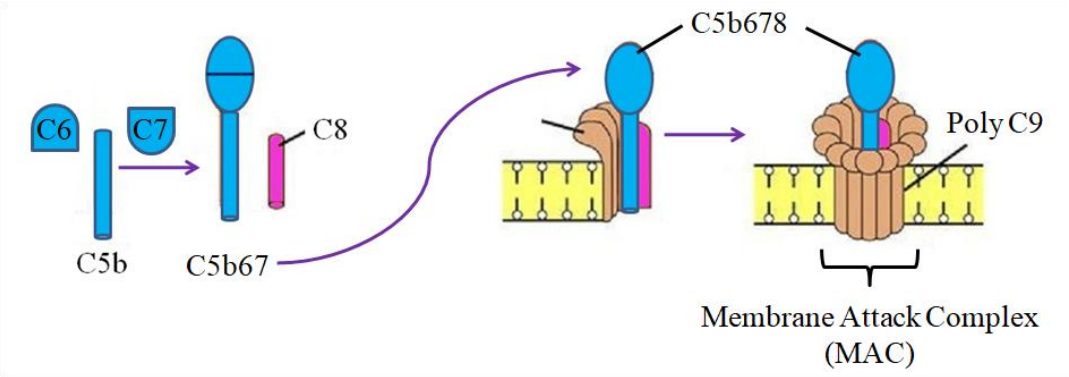
C-reactive protein is an acute phase reactant that can activate the classical pathway of the complement system, and its role in the complement led ischemia–reperfusion injury (IRI) has been shown in intestinal and myocardial animal IRI models. Similarly, cross-talk between complement and toll-like receptors has shown to be possible due to mitogen activated protein kinases in renal IRI setting.

Cross-talk between complement system and other systems will exist, and future research will be aimed at evaluating these ‘communicators’ between systems.

2.5 Complement Cascade - The Three Complement Pathways Converge At the Membrane-Attack Complex:

The principal function of the complement system is protection of the host from infection/inflammation by recruiting (chemotaxis) and enhancing phagocytosis by innate immune cells (opsonisation), leading to lysis of the target cells. All three pathways lead to the generation of C3 convertase that cleaves the C3 protein into C3a and C3b. While C3a acts as an anaphylatoxin, C3b covalently binds to the activating surface and participates in the self-activation loop of complement activation via the alternate pathway. C3b also associates with C3 convertases (C4b2a or C3bBb) to form the C5 convertase, which cleaves C5 complement into C5a and C5b. Interaction of C5b with C6, C7, C8 and C9 leads to formation of C5b–9/MAC (Membrane Attack Complex) (Fig. 2), a multimolecular structure that inserts into the membrane creating a functional pore leading to cell lysis.

MAC can cause lysis of some cells (e.g. erythrocytes) with a single hit, but some nucleated cells required multiple hits, or rather, multiple channel formation to cause cell lysis.



However, studies have shown that when the number of channels assembled on the cells is limited, sublytic C5b–9 can activate transcription factors and signal transduction, leading to inhibition of apoptosis and cell homeostasis.

Figure 2.2.: A Model for MAC Formation. The complement terminal pathway is initiated by the cleavage of C5 to C5b. C6 traps a labile conformation of the C5b TED (Thioester-containing domain) domain to form C5b6, a platform for the stepwise assembly of components C7, C8, and C9. Regulatory proteins in the plasma block MAC assembly in solution by binding exposed hydrophobic regions and sterically inhibit C9 oligomerization. Binding of C5b8 to membranes recruits multiple C9 molecules.

The anaphylatoxins (C3a and C5a) are key players in the recruitment of inflammatory cells and release of mediators that amplify the inflammatory response. C5a is probably the principal anaphylatoxin mediating inflammation. C5a binds to C5a receptor (C5aR or CD88) that is widely present on inflammatory and noninflammatory cells.

Apart from recruiting the neutrophils, C5a also increases neutrophil adhesiveness and aggregation. C5a causes secretion of pro-inflammatory cytokines and lysosomal enzymes from the macrophages and monocytes, thus leading to chemotaxis. C5a also up-regulates adhesion molecules such as α-integrin and β 2-integrin; in particular, Mac-1, in polymorphonuclear leukocytes. C5a was shown to be an important inflammatory mediator for the early adhesive interactions between neutrophils and endothelial cells in the acute inflammatory response.

It is responsible for up-regulation of vascular adhesion molecules such as P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [29, 72]. C3a does not act as a chemoattractant for neutrophils, but aids migration of eosinophils and mast cells. C3a and C5a also act on their receptors expressed on innate immune cells such as dendritic cells, thus playing a role in initiating and regulating T cell responses. In the IRI setting, MAC has been shown to mediate IR injury, and its inhibition was shown to attenuate the IRI effect.

2.6 Biological Consequences of Complement Activation:

Complement serves as an important mediator of the humoral response by amplifying the response and converting it into an effective defense mechanism to destroy invading microorganisms. The MAC mediates cell lysis, while other complement components or split products participate in the inflammatory response, opsonization of antigen, viral neutralization, and clearance of immune complexes (Table 1).

2.6.1 The Membrane-Attack Complex Can Lyse A Broad Spectrum Of Cells:

The membrane-attack complex formed by complement activation can lyse gram-negative bacteria, parasites, viruses, erythrocytes, and nucleated cells. Because the alternative and lectin pathways of activation generally occur without an initial antigen-antibody interaction, these pathways serve as important innate immune defenses against infectious microorganisms. The requirement for an initial antigen-antibody reaction in the classical pathway supplements these nonspecific innate defenses with a more specific defense mechanism. In some instances, the requirement for antibody in the activating event may be supplied by so-called natural antibodies, which are raised against common components of ubiquitous microbes. Nevertheless, antibody and complement do play a role in host defense against viruses and are often crucial in containing viral spread during acute infection and in protecting against reinfection. Most, perhaps all, enveloped viruses are susceptible to complement mediated lysis. The viral envelope is largely derived from the plasma membrane of infected host cells and is therefore susceptible to pore formation by the membrane attack complex. The complement system is generally quite effective in lysing gram-negative bacteria.

Gram-positive bacteria are generally resistant to complement-mediated lysis because the thick peptidoglycan layer in their cell wall prevents insertion of the MAC into the inner membrane. Although complement activation can occur on the cell membrane of encapsulated bacteria such as *Streptococcus pneumoniae*, the capsule prevents interaction between C3b deposited on the membrane and the CR1 on phagocytic cells. Lysis of nucleated cells requires formation of multiple membrane attack complexes, whereas a single MAC can lyse a red blood cell. Many nucleated cells, including the majority of cancer cells, can endocytose the MAC.

2.6.2 Cleavage Products of Complement Components Mediate Inflammation:

The complement cascade is often viewed in terms of the final outcome of cell lysis, but various peptides generated during formation of the MAC play a decisive role in the development of an effective inflammatory response. The smaller fragments resulting from complement cleavage, C3a, C4a, and C5a, called anaphylatoxins, bind to receptors on mast cells and blood basophils and induce degranulation, with release of histamine and other pharmacologically active mediators. The anaphylatoxins also induce smooth-muscle contraction and increased vascular permeability. Activation of the complement system thus results in influxes of fluid that carries antibody and phagocytic cells to the site of antigen entry. C3a, C5a, and C5b67 can each induce monocytes and neutrophils to adhere to vascular endothelial cells, extravasate through the endothelial lining of the capillary, and migrate toward the site of complement activation in the tissues.

2.6.3 C3b and C4b Binding Facilitates Opsonization:

C3b is the major opsonin of the complement system, although C4b also has opsonizing activity. The amplification that occurs with C3 activation results in a coating of C3b on immune complexes and particulate antigens. Phagocytic cells, as well as some other cells, express complement receptors (CR1, CR3, and CR4) that bind C3b or C4b. Antigen coated with C3b binds to cells bearing CR1. If the cell is a phagocyte (e.g., a neutrophil, monocyte, or macrophage), phagocytosis will be enhanced. Activation of phagocytic cells by various agents, including C5a anaphylatoxin, has been shown to increase the number of CR1s on activated cells, greatly facilitating their phagocytosis of C3b-coated antigen.

Recent studies indicate that complement fragment C3b acts as an adjuvant when coupled with protein antigens.

C3b targets the antigen directly to the phagocyte, enhancing the initiation of antigen processing and accelerating specific antibody production.

Table 2.1: Summary of biological effects mediated by complement products.

Effect	**Complement product mediating***
Cell lysis	C5b–9, the membrane-attack complex (MAC)
Inflammatory response	
Degranulation of mast cells and basophils†	C3a,C4a, and C5a (anaphylatoxins)
Degranulation of eosinophils	C3a, C5a
Extravasation and chemotaxis of leukocytes at inflammatory site	C3a, C5a, C5b67
Aggregation of platelets	C3a, C5a
Inhibition of monocyte/macrophage migration and induction of their spreading	Bb
Release of neutrophils from bone marrow	C3c
Release of hydrolytic enzymes from neutrophils	C5a
Increased expression of complement receptors type 1 and 3 (CR1 and CR3) on neutrophils	C5a
Opsonization of particulate antigens, increasing their phagocytosis	C3b, C4b
Viral neutralization	C3b, C5b–9 (MAC)
Solubilization and clearance of immune complexe	C3b
*Boldfaced component is most important in mediating indicated effect.	
†Degranulation leads to release of histamine and other mediators that induce contraction of smooth muscle and increased permeability of vessels.	

2.6.4 The Complement System Also Neutralizes Viral Infectivity:

For most viruses, the binding of serum antibody to the repeating subunits of the viral structural proteins creates particulate immune complexes ideally suited for complement activation by the classical pathway. Some viruses (e.g., retroviruses, Epstein-Barr virus, Newcastle disease virus, and rubella virus) can activate the alternative, lectin, or even the classical pathway in the absence of antibody. The complement system mediates viral neutralization by a number of mechanisms. Some degree of neutralization is achieved through the formation of larger viral aggregates, simply because these aggregates reduce the net number of infectious viral particles. Although antibody plays a role in the formation of viral aggregates, in vitro studies show that the C3b component facilitates aggregate formation in the presence of as little as two molecules of antibody per virion. The binding of antibody and/or complement to the surface of a viral particle creates a thick protein coating. This coating neutralizes viral infectivity by blocking attachment to susceptible host cells. The deposits of antibody and complement on viral particles also facilitate binding of the viral particle to cells possessing Fc or type 1 complement receptors (CR1). In the case of phagocytic cells, such binding can be followed by phagocytosis and intracellular destruction of the ingested viral particle. Finally, complement is effective in lysing most, if not all, enveloped viruses, resulting in fragmentation of the envelope and disintegration of the nucleocapsid.

2.6.5 The Complement System Clears Immune Complexes From Circulation:

The importance of the complement system in clearing immune complexes is seen in patients with the autoimmune disease systemic lupus erythematosus (SLE). These individuals produce large quantities of immune complexes and suffer tissue damage as a result of complement-mediated lysis and the induction of type II or type III hypersensitivity. Although complement plays a significant role in the development of tissue damage in SLE, the paradoxical finding is that deficiencies in C1, C2, C4, and CR1 predispose an individual to SLE; indeed, 90% of individuals who completely lack C4 develop SLE. The complement deficiencies are thought to interfere with effective solubilization and clearance of immune complexes; as a result, these complexes persist, leading to tissue damage by the very system whose deficiency was to blame.

The coating of soluble immune complexes with C3b is thought to facilitate their binding to CR1 on erythrocytes. Although red blood cells express lower levels of CR1 (~5 X 10^2 per cell) than granulocytes do (~5 X 10^4 per cell), there are about 10^3 red blood cells for every white blood cell; therefore, erythrocytes account for about 90% of the CR1 in the blood. For this reason, erythrocytes play an important role in binding C3b-coated immune complexes and carrying these complexes to the liver and spleen. In these organs, immune complexes are stripped from the red blood cells and are phagocytosed, thereby preventing their deposition in tissues. In SLE patients, deficiencies in C1, C2, and C4 each contribute to reduced levels of C3b on immune complexes and hence inhibit their clearance. The lower levels of CR1 expressed on the erythrocytes of SLE patients also may interfere with the proper binding and clearance of immune complexes.

2.7 Complement Deficiency:

Genetic deficiencies have been described for each of the complement components. Homozygous deficiencies in any of the early components of the classical pathway (C1q, C1r, C1s, C4, and C2) exhibit similar symptoms, notably a marked increase in immune-complex diseases such as systemic lupus erythematosus, glomerulonephritis, and vasculitis. These deficiencies highlight the importance of the early complement reactions in generating C3b, and the critical role of C3b in solubilization and clearance of immune complexes. In addition to immune complex diseases, individuals with such complement deficiencies may suffer from recurrent infections by pyogenic (pusforming) bacteria such as streptococci and staphylococci. These organisms are Gram-positive and therefore resistant to the lytic effects of the MAC. Nevertheless, the early complement components ordinarily prevent recurrent infection by mediating a localized inflammatory response and opsonizing the bacteria. Deficiencies in factor D and properdin, early components of the alternative pathway, appear to be associated with Neisseria infections but not with immune-complex disease. Patients with C3 deficiencies have the most severe clinical manifestations, reflecting the central role of C3 in activation of C5 and formation of the MAC. Individuals with homozygous deficiencies in the components involved in the MAC develop recurrent meningococcal and gonococcal infections caused by *Neisseria* species. In normal individuals, these gram-negative bacteria are generally susceptible to complement-mediated lysis or are cleared by the opsonizing activity of C3b.

MAC-deficient individuals rarely have immune-complex disease, which suggests that they produce enough C3b to clear immune complexes.

Interestingly, a deficiency in C9 results in no clinical symptoms, suggesting that the entire MAC is not always necessary for complement-mediated lysis. Congenital deficiencies of complement regulatory proteins have also been reported.

The C1 inhibitor (C1 Inh) regulates activation of the classical pathway by preventing excessive C4 and C2 activation by C1.

The deficiency gives rise to a condition called hereditary angioedema, which manifests clinically as localized edema of the tissue, often following trauma, but sometimes with no known cause.

Investigations of in vivo complement activity in these animals has allowed dissection of the complex system of complement proteins and the assignment of precise biologic roles to each.

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