Diagnostic Immunology Laboratory Manual

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Preface

This manual is intended for laboratories involved in the assessment of primary and secondary diseases of the host defense and immune systems. Whether this is a diagnostic immunology laboratory or a research laboratory with an interest in host defense and human immune system disorders, this manual will provide a methodological base for evaluating the components of these systems. The procedures described cover most of the major aspects of these two systems and how each of these components can be analyzed.

This manual has been written and designed for the technologist or clinical immunology laboratory scientist to use at the laboratory work bench. The techniques have been in use in the authors' laboratories for several years and special consideration has been given to discussing issues and problems that the laboratory worker may encounter when performing each of the techniques. This manual provides sufficient detail so that the first time through the procedure should be successful. It would be impossible to incorporate into one manual all of the methods for carrying out a procedure. Hence this manual provides in great detail the basic procedures that the authors have found to be useful in their laboratory while providing comments concerning other procedures or alternative methods, as well as references for those who wish to extend their investigations.

The sections in this manual follow the recommendations of the National Committee on Clinical Laboratory Standards (NCCLS) guidelines for a clinical laboratory procedure manual (document GP2-A). Each procedure contains the following: a section on the patient preparation; the specimen to be collected; how the specimen should be handled; the principle underlying the technique and the clinical indications for performing the test; all reagents, standards, controls, and equipment that one needs; a step by step procedure; a description of the calculations or how the results are obtained; an unacceptable results section; normal ranges; an additional remarks section which addresses potential

pitfalls or alternative methods that may not have been addressed elsewhere in the procedure; and a list of major references.

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1

Background and General Information for Complement Assays

The following information is not intended as an in-depth review of complementology but rather as an overview of some basics, with emphasis on applying the assays described to the analysis of complement in the laboratory. There are references listed at the end of this chapter that will provide the reader with more details about the biochemistry, genetics, and biology of complement.

Complement is an enzyme cascade system composed of about 20 proteins that are present in the circulation as precursor enzymes, enzyme cofactors, control proteins, and effector molecules (Table 1). There are also receptors for complement components on white and red blood cells, on many cells of the reticuloendothelial system, and on other tissue cells, such as mast cells. The interaction of complement proteins with these cells contributes to inflammation as well as modulating immune responses to certain antigens.

Complement can be activated by two distinct pathways (classical and alternative) as well as by coagulation and cell-derived enzymes. In general, the first step in either the classical or the alternative pathway involves recognition of a complement-activating substance (Table 2), with subsequent binding of complement proteins to the activator. This binding allows the formation of the first macromolecular enzyme complex and triggers the enzyme cascade in which complement split products are produced. The final step of complement activation is the assembly of the membrane attack complex (MAC) on the surface of the activating particle. If the activator is a microorganism or an antibody-coated cell, the result of MAC formation may be lysis of the cell. Figure 1 summarizes this three-part activation process and shows where the individual components fit into this scheme.

TABLE 1. *Components of the human complement system*

Component symbol	Concentration in serum (μg/ml)[a]	Molecular weight (daltons)	Electrophoretic mobility	Associated pathway	Enzymatic activity[b]
C1q	180	400,000	gamma-2	Classical	No
C1r	101	92,000	beta	Classical	Yes
C1s	110	86,000	alpha	Classical	Yes
C2	25	117,000	beta-1	Classical	Yes
C4	640	206,000	beta-1	Classical	No
C3	1000	185,000	beta-2	Classical and alternative	No
C3b	Trace	170,000	beta-1	Alternative	No
C5	80	180,000	beta-1	Terminal	No
C6	75	95,000	beta-2	Terminal	(?)
C7	55	110,000	beta-2	Terminal	No
C8	80	163,000	beta-2	Terminal	No
C9	50	79,000	alpha-2	Terminal	No
D	2	25,000	alpha	Alternative	Yes[b]
B	200	93,000	beta	Alternative	Yes
P	25	184,000	gamma-2	Alternative	No
C1-Inh	25	90,000	gamma-2	Control	No
I	25	100,000	beta-2	Control	Yes[b]
H	500	150,000	beta-1	Control	No
C4bp	250	550,000	beta	Control	No
CFI	<10	310,000	alpha-1	Control	Yes
S	200	80,000	alpha	Control	No
C3a	<0.6	11,000	anodal	Split product	No
C4a	<1.5	12,000	anodal	Split product	No
C5a	<0.01	11,000	anodal	Split product	No
Bb	<0.6	60,000	gamma	Split product	Yes
SC5b-9	<0.6	1,000,000		MAC	No

[a]Average for adults.
[b]Most complement enzymes exist as precursors, but factors D and I are enzymatically active in the circulation.

Once the initiation of complement activation has occurred, the subsequent steps depend on the type and amount of the activator, its location, and the concentrations of the individual complement components and control proteins. The two major pathways of complement activation, the classical and the alternative, are distinguished by the complement components involved in their initiation sequence, the types of substances that activate them, and their requirements for calcium ions, magnesium ions, or both. In addition to these pathways, enzymes, such as those from the coagulation and kinin-generating systems and from inflammatory cells, such as neutrophils and macrophages, also can produce biologically active complement fragments. Soluble activators, such as small immune complexes and low molecular weight substances, tend to activate the early components and spare the late components, whereas large particles, such as bacteria, antibody-coated cells, aggre-

TABLE 2. *Partial list of substances that activate complement*

Substance	Complement pathway activated
Antigen-antibody complexes	Classical
DNA, polyinosinic acid	Classical
Polyanion-polycation complexes (heparin-protamine sulfate)	Classical
C-reactive protein complexes	Classical
Enveloped viruses (some)	Classical
Monosodium urate crystals	Classical
Lipid A of bacterial lipopolysaccharide	Classical
Plicatic acid	Classical
Ant venom polysaccharide	Classical
Inulin	Alternative
Yeast cell walls (zymosan)	Alternative
Sephadex	Alternative
Endotoxin (bacterial lipopolysaccharide)	Alternative
Rabbit erythrocytes	Alternative
Desialylated human erythrocytes	Alternative
Cobra venom factor	Alternative
Aggregated immunoglobulins	Classical and alternative
Subcellular membranes (mitochondria)	Classical and alternative
Cell- and plasma-derived enzymes Plasmin, kallikrein, activated Hageman factor (XII), neutrophil elastase	Classical, alternative, terminal

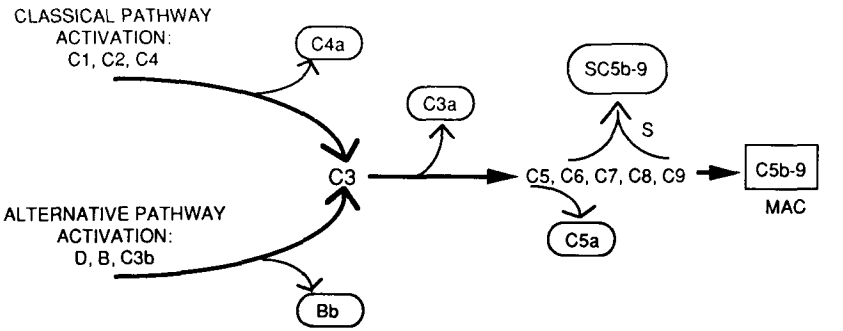

FIG. 1. Complement cascade showing split products.

Recognition of Activating Substance → Macromolecular Enzyme Formation → Biological Activity: Lysis, Chemotaxis, †Vascular Permeability, †Phagocytosis

gated immunoglobulins, or complex polysaccharides, may consume large amounts of complement. In the former case, the tests normally done to evaluate complement (CH50, C3, C4) may not detect a significant change. New technology based on monoclonal antibodies specific for complement split products has made it possible to detect activation of less than 1% of the complement in the circulation, close to the range that includes normal turnover of complement components. Thus, small fluctuations of complement activation can be detected, allowing the physician to closely monitor patients for early warning of flares of immune complex diseases or other complement-activating processes.

Disorders of the complement system can be either hereditary or acquired and can have profound impacts on the expression of disease in the affected individual. Hereditary complement deficiencies have been described for all of the complement components and usually result in complete absence of a single component from an individual's serum. Exceptions are C1-Inh and C8 deficiency which are defined on the basis of decreased levels of the proteins accompanied by loss of function. Acquired deficiencies can result from activation and consumption of complement by a disease process, decreased synthesis, and increased catabolism (4). Careful analysis of complement can distinguish between these different causes of complement disorders.

MEASUREMENT OF COMPLEMENT

The two usual reasons for ordering complement tests when evaluating a patient's immune system are

1. To determine whether complement activation is occurring in vivo as a result of infection, autoimmune processes, drug treatment, or other mechanisms
2. To determine whether the patient has a hereditary complement deficiency that might be related to his or her clinical condition

The series of tests described in this section should make it easier to address these questions.

Tests for Complement Activation

Low-level complement activation may be a good indicator of disease activity but cannot always be documented by looking at CH50 or C3 or C4. These assays lack sensitivity, and an acute phase response may mask decreases due to activation. The best procedure is to look at fragments of the complement proteins that are produced only when complement

activation occurs. An added advantage is that by measuring some of the split products produced during complement activation, one can determine whether activation is occurring via the classical or the alternative pathway and how complete the activation process is. There are commercially available enzyme immunoassays (EIA) (for C4d, iC3b, Bb, and SC5b-9 from Quidel, San Diego, CA) and radioimmunoassays (RIA) (for C3a, C4a, and C5a from Amersham, UK) that can quantitate some of the complement component split products.

Classical	Alternative	Terminal
C4a	Ba	C3a, C3d
C4d	Bb	iC3b
C4/C4d ratio		C5a, SC5b-9

These assays are sensitive enough to detect activation of as little as 1% of the complement in serum. Specimen requirements are 1 mL EDTA or heparin-plasma, stored at −70°C if the time delay after collection is greater than 4 hours. *Note:* Serum will give false positive results due to split products created by the coagulation process.

Tests To Detect Complement Deficiencies

The initial screening of serum for complement deficiency should include both CH50 and AH50 assays to cover the classical and alternative pathways. If a deficiency exists, its location in the cascade can be determined from results of these assays as shown in the following table.

CH50	AH50	Location of Defect
Normal	Normal	None
Normal	Absent or very low	Alternative pathway (B,P)
Absent or very low	Normal	Classical pathway (C1, C2, C4)
Absent or very low	Absent or very low	Terminal pathway (C3, C5, C6, C7, C8, C9)

Low, but not absent, levels of CH50 and AH50 activity could be the result of activation or of a heterozygous complement deficiency state. Once the CH50 and AH50 results are known, the exact defect can be determined by examining individual components of the affected pathway. Either functional assays or measurements of the protein levels will determine the missing protein. A rapid functional screen for C5, C6,

C7, C8, and C9 provides qualitative results for late component screening. C8 deficiency should be confirmed by functional assay because C8-deficient individuals have immuno-reactive protein that gives false positive levels.

The specimen requirements for complement deficiency evaluation are a minimum of 1 mL serum, separated from the clot within 2 hours of blood drawing, and kept frozen at −70°C if not assayed immediately. *Note:* Incorrect specimen handling (repeated freeze–thaw, room temperature for more than 1 to 2 hours, freezing or storage at temperatures above −70°C for longer than 24 hours), as well as collection of the wrong type of specimen (i.e., plasma instead of serum), could result in decreased complement activity and misinterpretation of results (1–3).

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SECTION I

Assessment of Complement Components and Their Function