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# A quantitative lateral flow assay to detect complement activation in blood

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ABSTRACT

Complement is a major effector arm of the innate immune system that responds rapidly to pathogens or altered self. The central protein of the system, C3, participates in an amplification loop that can lead to rapid complement deposition on a target and, if excessive, can result in host tissue damage. Currently, complement activation is routinely monitored by assessing total C3 levels, which is an indirect and relatively insensitive method. An alternative approach would be to measure downstream C3 activation products such as C3a and iC3b. However, in vitro activation can produce falsely elevated levels of these biomarkers. To circumvent this issue, a lateral flow immunoassay system was developed that measures iC3b in whole blood, plasma, and serum and avoids in vitro activation by minimizing sample handling. This assay system returns results within 15 min and specifically measures iC3b while having minimal cross-reactivity to other C3 split products. While evaluating the potential of this assay, it was observed that circulating iC3b levels can distinguish healthy individuals from those with complement activation-associated diseases. This tool is engineered to provide an improved method to assess complement activation at point of care and could facilitate studies to monitor disease progression in a variety of inflammatory conditions.

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The complement system is a phylogenetically ancient branch of the innate immune system that primarily serves to eliminate foreign pathogens from the host [1,2]. A second function of the complement system is to recognize and mark altered self, such as apoptotic or necrotic cells, for clearance [3]. The complement system is activated via three distinct pathways: classical, lectin, and alternative. Whereas the classical and lectin pathways are initiated by antibodies recognizing antigens and lectins binding sugars, respectively, the alternative pathway (AP)[1] is spontaneously triggered at a continuous low rate in the blood through a process known as "tick over" (reviewed in Ref. [4]). The three activation cascades converge at the central step of activation of component 3 (C3) (Fig. 1). In addition, recent studies have identified an extrinsic pathway that allows activation of C3 (and the downstream component 5 [C5]) via enzymes of the coagulation pathway and other proteases [5,6].

C3 is the most abundant protein of the complement system and its key opsonic protein [7]. Proteolytic activation of C3 leads to two split products, C3a and C3b. Deposition of C3b marks pathogens and waste material for clearance by phagocytic cells through immune adherence and ingestion [3]. Release of the anaphylatoxins C3a and the downstream C5a leads to recruitment of inflammatory cells such as neutrophils to a site of infection. Furthermore, initiation of the terminal pathway leads to membrane perturbation and subsequent target cell lysis by the membrane attack complex (MAC).

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[1] *Abbreviations used:* AP, alternative pathway; C3, component 3; CR2/3/4, complement receptor 2/3/4; SLE, systemic lupus erythematosus; LFA, lateral flow assay; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; RT, room temperature; ICH, intracerebral hemorrhage; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

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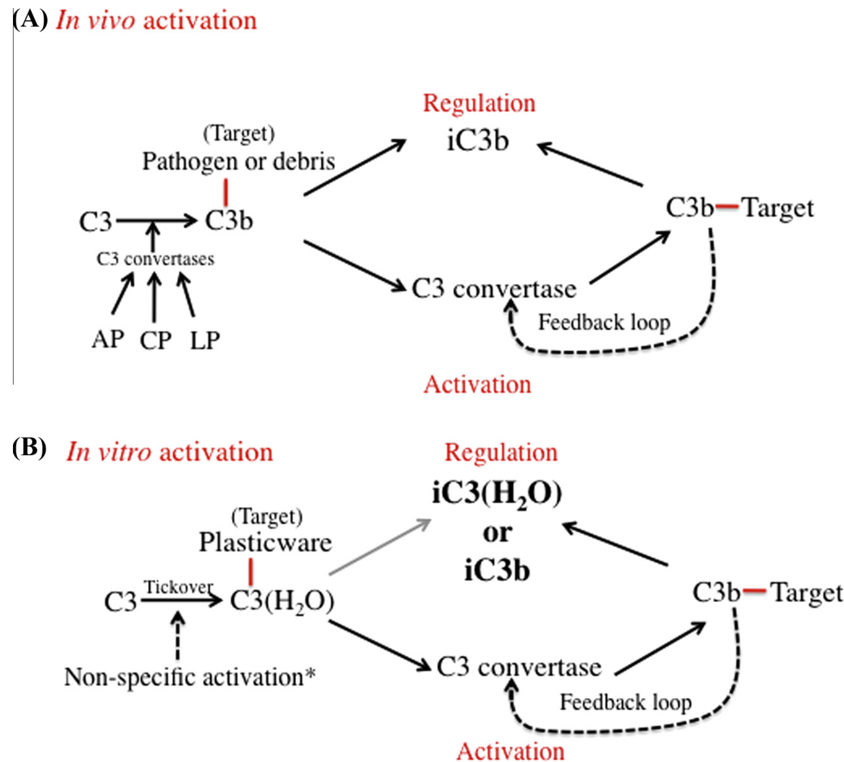


**Fig.1.** Diagram of C3 activation. (A) In vivo C3 is commonly activated to C3b via the C3 convertases, after which it can covalently attach to a target. The resulting C3b may serve as an opsonin as well as participate in an amplification loop or be inactivated via regulatory proteins. (B) In vitro C3 activation may occur during exposure to plasticware, improper storage, freeze–thaw cycles, or activity of clotting or other proteolytic enzymes. In vitro activation leads to more C3b generation, and then this may be amplified. See introductory paragraphs for further explanation. AP, alternative pathway; CP, classical pathway; LP, lectin pathway; red line, covalent bond. (For interpretation of the reference to color in this figure legend, the reader is referred to the Web version of this article.)

C3 is activated by the C3 convertases, enzymatic complexes formed via all three pathways that convert C3 to C3a and C3b (Fig. 1A). During this conversion, the protein undergoes a dramatic conformational change that results in exposure of the thioester bond [8,9]. This is a highly reactive species that enables the transfer of the protein from the fluid phase to nearby targets through a covalent interaction. C3b is itself a component of the AP C3 convertase and, thus, participates in its own activation. This results in a powerful positive feedback loop (the AP amplification loop) that can result in the rapid deposition of many copies of C3b on a target (reviewed in Ref. [10]).

To prevent damage to the host, complement activation is stringently regulated (Fig. 1A). Once produced, C3b can be rapidly converted to iC3b by the serine protease factor I (FI) and a cofactor protein that releases a small fragment of 18 amino acids, C3f, into the fluid phase (reviewed in Refs. [11,12]). iC3b cannot participate in the amplification loop of the AP and is normally cleared from circulation.

Although iC3b does not participate in the complement activation cascade, it does have important immunological roles. iC3b binds to complement receptors 2, 3, and 4 (CR2, CR3, and CR4). Upon binding to CR2 on B-cells, iC3b contributes to the humoral response by serving as a costimulatory signal. CR3 and CR4 are expressed on myeloid cells, particularly neutrophils and macrophages, respectively. When iC3b engages either of these receptors, it leads to adhesion as well as phagocytosis. Deficiency of CR3/CR4 leads to leukocyte adhesion deficiency syndrome (reviewed in Ref. [13]).

The initiating C3 convertase [$C3(H_2O)Bb$] of the AP is thought to be generated through the tick-over process. This system continuously surveys the host environment for danger and, if found, initiates a response within seconds. When generated, C3b deposits on any nearby surface with nucleophilic groups, whether it is a foreign surface or self [14]. In healthy individuals, there is a balance between activation and regulation that allows for constant surveillance for targets while preventing excessive activation on healthy host cells. However, if imperfectly regulated, the system also allows for chronic nonspecific activation and can contribute to the pathology of chronic inflammatory disease [4]. If C3 comes in contact with nonbiological surfaces such as dialysis tubing and plasticware used in the laboratory, it can deposit on those surfaces as well, causing further in vitro activation through the feedback loop and shifting the balance toward further C3b generation (Fig. 1B). Similarly, if samples are not handled carefully or stored properly, in vitro activation can occur and may mask the in vivo signal, cause falsely elevated levels of split products, and confound interpretation of the patient's complement activation status.

Complement activation is associated with many diseases, including systemic lupus erythematosus (SLE) [15], atypical hemolytic uremic syndrome (aHUS) [16], membranoproliferative glomerulonephritis I (MPGN I) [17], dense deposit disease (DDD) [18,19], age-related macular degeneration (AMD) [20], myocardial infarction [21], and preeclampsia (PE) [22]. Current clinical tests for complement measure either functional activity by total hemolytic activity (CH50) or total C3 antigen levels (intact C3 plus activation breakdown products) with an immunoassay. Although these tests are established and used routinely, they might not accurately reflect C3 activation status and lack the sensitivity to identify lower levels of activation. The normal range for both functional assessments and immunoassays is fairly wide; therefore, a substantial change is required before C3 activation is reliably detected by these measures. Because C3 is an acute phase reactant, during times of inflammation C3 may be consumed while production by the liver is increased. Consequently, a continuous moderate

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