Molecular Immunology 114 (2019) 353–361

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Is generation of C3(H_2O) necessary for activation of the alternative pathway in real life?

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ARTICLE INFO

Keywords:
Complement system
C3(H_2O)
Conformation
Analysis
Proteases
Alternative pathway

ABSTRACT

In the alternative pathway (AP) an amplification loop is formed, which is strictly controlled by various fluid-phase and cell-bound regulators resulting in a state of homeostasis. Generation of the "C3b-like" C3(H_2O) has been described as essential for AP activation, since it conveniently explains how the initial fluid-phase AP convertase of the amplification loop is generated. Also, the AP has a status of being an unspecific pathway despite thorough regulation at different surfaces.

During complement attack in pathological conditions and inflammation, large amounts of C3b are formed by the classical/lectin pathway (CP/LP) convertases. After the discovery of LP´s recognition molecules and its tight interaction with the AP, it is increasingly likely that the AP acts *in vivo* mainly as a powerful amplification mechanism of complement activation that is triggered by previously generated C3b molecules initiated by the binding of specific recognition molecules.

Also in many pathological conditions caused by a dysregulated AP amplification loop such as paroxysmal nocturnal hemoglobulinuria (PNH) and atypical hemolytic uremic syndrome (aHUS), C3b is available due to minute LP and CP activation and/or generated by non-complement proteases. Therefore, C3(H_2O) generation *in vivo* may be less important for AP activation during specific *attack* or dysregulated *homeostasis*, but may be an important ligand for C3 receptors in cell-cell interactions and a source of C3 for the intracellular complement reservoir.

1. Introduction of the complement system

The complement system can be activated by three different pathways: the classical (CP), the lectin (LP), and the alternative pathways (AP). Recognition molecules within these pathways bind and initiate the assembly of the C3 convertases C4bC2a (CP and LP) and C3bBbP (AP), both of which cleave C3 between Arg^{726} and Ser^{727} in the α-chain (de Bruijn and Fey, 1985) thereby generating the opsonin C3b and the anaphylatoxin C3a (Bokisch et al., 1969). C3a is released into the fluid phase and the opsonin C3b can bind to pathogens or other target surfaces (Fig. 1). However, opsonization by convertase-generated C3b is a rather inefficient process since typically less than 5% of the available C3 ends up covalently attached to a target surface (Ekdahl and Nilsson, 1999; Nilsson and Nilsson, 1985). This implies that the residual C3b molecules remain in solution, because the thiol ester reacts with available nucleophiles in solution, mainly H_2O.

Complement activation at the C3-level is regulated by multiple soluble and cell-bound inhibitors, many of which act as co-factors to factor I in order to promote proteolytic cleavage of the C3b and C4b components of the convertases. The generated C3 fragments can separately or in concert lead to lysis and mediate chemotaxis, phagocytosis, cytotoxicity, immunomodulation etc. via interaction with the complement receptors preferentially on leukocytes. The AP is a complex sequence of events that has been carefully studied, but the mechanism of AP activation is still not fully understood which partly may be due to that many of the studies are biased by methodological issues *in vitro*. One of those is that complement has traditionally been studied in blood serum where much of the coagulation system factors are activated and another one is that the AP has been studied in the presence of EGTA which chelates Ca^{2+} and prevents both CP and LP activation. This hampers studies of possible cross-talks between different parts of the complement system and with other cascade systems. In recent years,

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https://doi.org/10.1016/j.molimm.2019.07.032
Received 27 May 2019; Received in revised form 18 July 2019; Accepted 31 July 2019
Available online 22 August 2019

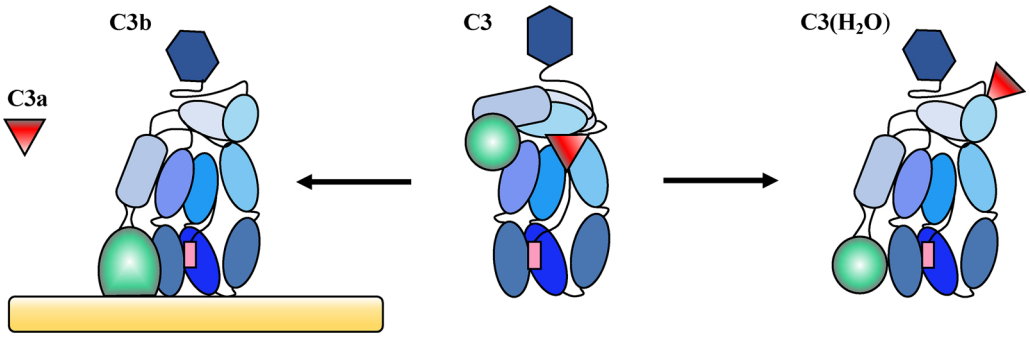

Fig. 1. Conformational changes in complement component C3. Native C3 (middle) gets cleaved by the C3 convertases which leads to formation of C3b and release of C3a (red; left). Alternatively, C3 undergoes hydrolysis to C3(H_2O) which still contains the C3a domain but which points out from the rest of the molecule thereby exposing neo-epitopes (right) which are not seen in native C3 (middle). In both cases, the formation of C3b and C3(H_2O) involves translocation of the thiolester-ester containing domain (green) which enables C3b to form covalent bonds with −OH or -NH2 residues on the target surface (left). The figure is adapted from (Gros et al., 2008) with permission from the publisher. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

some of these cross-talk mechanisms have been unraveled and suggest that additional mechanism may be operative. The present review discusses possible concepts that are involved in the activation of the AP.

2. C3 structure

C3 consists of 11 domains, 8 of which are so called macroglobulin domains (Janssen et al., 2005). It contains a unique thiol ester, initially characterized by Prahl and coworkers (Janatova et al., 1980a, 1980b) which is located between Cys^{988} and Gln^{991} in the thiol ester domain (TED) (Fey et al., 1984). The thiol ester facilitates covalent binding of C3b to hydroxyl (−OH) and amine (–NH_2) groups (Law and Levine, 1977; Law et al., 1981) on target surfaces and is destroyed by attack by nucleophiles (H_2O, NH_3, methylamine etc), either located on the target surface or in the fluid phase (Pangburn and Müller-Eberhard, 1980; Pangburn et al., 1981), generating C3(H_2O) (Fig. 1).

In this review article, we refer to all forms of C3 with a disrupted thiol ester as "C3(H_2O)" regardless of the nature of the attacking nucleophile (H_2O, methylamine etc). The rate of this hydrolysis can be substantially accelerated by chaotropic and denaturing agents e.g. KSCN, urea, guanidine HCl, and charged detergents, e.g., SDS (Pangburn and Müller-Eberhard, 1980). C3 with a broken thiol ester cannot be activated to C3a and C3b by the two convertases (Pangburn and Müller-Eberhard, 1980) or by trypsin. Except at nonphysiological conditions (i.e., high concentration and temperature, and prolonged incubation time trypsin digestion of C3(H_2O) yields C3a later in the cleavage sequence, i.e., concomitant with C3c and C3d (Nilsson and Ekdahl, unpublished data). Like C3b, C3(H_2O) is cleaved by factor I in the presence of a co-factor, e.g., factor H, to iC3(H_2O) at the same locations (Fig. 2) in the α-chain as in C3b but at a slower rate (Isenman et al., 1981).

3. Activation of the AP and the tick-over mechanism(s)

3.1. The importance of the initial deposition of C3b in the activation of the AP

The AP is initiated by C3b and factor B which form a complex that is dependent on the presence of Mg^{2+} as reviewed in (Lachmann, 2009). This initial complex formation is followed by the cleavage of factor B by factor D into Ba and Bb to form an active labile enzymatic complex C3bBb, the AP C3 convertase, which in the fluid phase has a half-life time of 90 s as determined *in vitro* using purified components (Fishelson et al., 1984; Pangburn and Müller-Eberhard, 1986). The C3bBb convertase can then cleave native C3 molecules into C3a and C3b. These C3b molecules trigger a positive feedback loop reaction, with each new C3b molecule potentially being able to form a new AP convertase complex. Theoretically, only one initial C3b molecule that starts the positive feedback loop is needed to commence the AP activation.

The anaphylatoxin C3a/$C3a_{desArg}$ is constantly generated with a half-life in plasma of approximately 30 min (Norda et al., 2012). If the concentration of C3 is increased, the C3a levels are proportionally raised (i.e, the C3a/C3 ratio is constant) which can be shown in a normal/obese population exhibiting a wide range in their blood plasma C3 concentration (B. Nilsson et al., 2014). This turn-over of C3 has been explained by the tick-over theory, put forward by Lachmann in the early 1970s (Lachmann and Nicol, 1973; Nicol and Lachmann, 1973). This theory states that low amounts of C3b are generated in sufficient quantity to be able to interact with factor B and initiate a fluid-phase convertase. The origin and configuration of this C3 species have not yet been fully elucidated. Furthermore, since C3 is susceptible to cleavage by a number of non-canonical proteases in addition to the two established C3 convertases, it might be digested to C3b (and C3a) by various proteolytic active enzymes in the plasma or in other body fluids.

3.2. The involvement of C3(H_2O) in the initiation of the AP

In the early 1980s Pangburn and coworkers described the continuous hydrolysis of the internal thiol ester in C3, generating a "C3b-like" molecule (Pangburn et al., 1981; Pangburn and Müller-Eberhard, 1980). Based on these findings, the tick-over of native C3 to C3(H_2O) has been the prevailing mechanism explaining the tick-over theory and activation of the AP. Here, we would like to discuss other possibilities than C3(H_2O) generation as the main mechanism by which the initial AP convertase is formed. Instead we propose, as originally was suggested by Lachmann and collaborators (Lachmann and Nicol, 1973; Nicol and Lachmann, 1973) that other mechanisms may be involved, such as solitary proteases, CP and LP activation, and/or interactions with surface interfaces.

In the initial study, the rate at which the thiol ester was hydrolyzed was estimated to be 0.2-0.4% per hour (Pangburn et al., 1981) which has been more or less confirmed in later studies using specific ELISAs (Elvington et al., 2019; Nilsson Ekdahl et al., 1992). The hemolytic activity of this form of C3 was zero, but instead the functions of C3b were conveyed to the molecule (Fig. 1). It was shown that C3(H_2O) exhibited C3b-like activity by that it can bind factor B and form an AP convertase (Fig. 3, right) and that it is cleaved by factor I in the presence of factor H.

A crucial question is how "C3b-like "is C3(H_2O)? A caveat in these early studies is that in all the presented experiments, the reaction was amplified either by C3 nephritic factor (C3Nef), properdin or by using Ni^{2+} instead of Mg^{2+}. C3Nef is an autoantibody that prevent decay of the AP convertase (Daha et al., 1976). It was also later shown that purified properdin preparations contain a large fraction of non-physiological aggregates (P_n), which can be separated by chromatography from the physiological forms (P_2, P_3 and P_4). Furthermore, only the aggregates caused fluid phase complement consumption when added to serum (Pangburn, 1989). More recently, the non-physiological activity of the aggregates of properdin was confirmed since P_n and unseparated properdin was shown to bind to numerous surfaces, in contrast to P_2-P_4

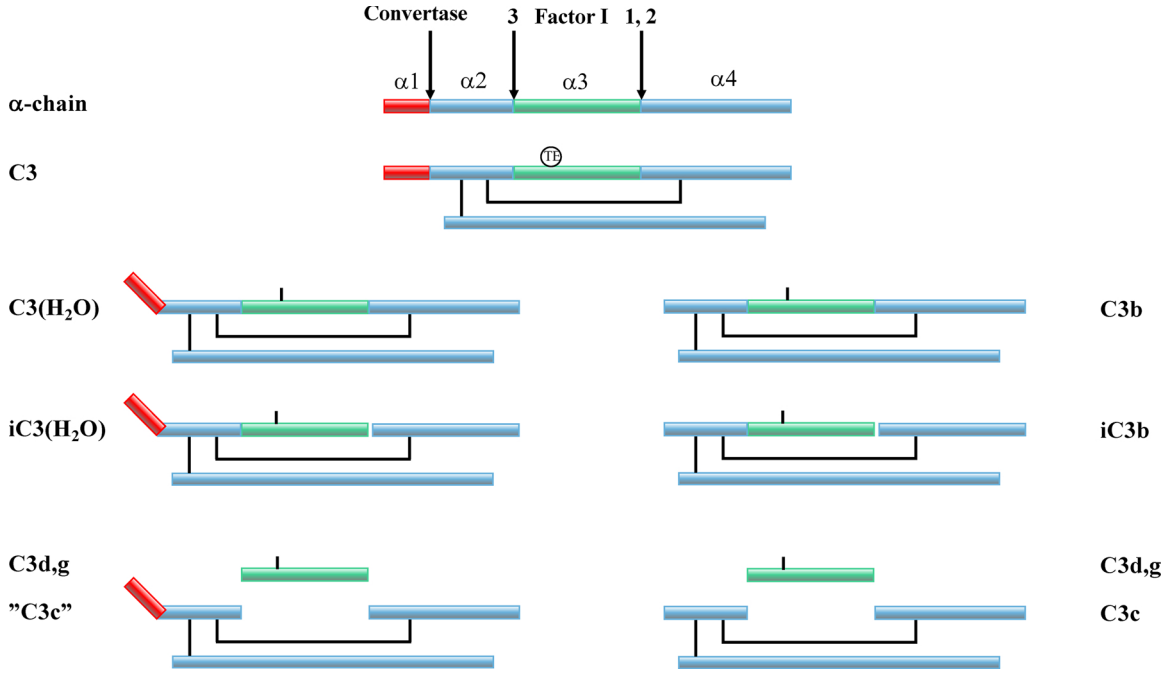

Fig. 2. Linear structures of C3, C3(H_2O) and proteolytic activation fragments. Schematic representation of the linear structure of the α-chain of C3 comprising the peptide sequences α1, α2, α3, α4, and indication of cleavage sites for the C3 convertases and factor I. In the intact C3 molecule, the disulfide bridges between the α- and β-chains and between α2 and α4 are indicated as well at the position of the thiol ester (TE; top). Disruption of the TE can induce formation of non-proteolytically activated C3(H_2O), which still contains the C3a-moiety and which can be digested by factor I: at the adjacent 1st and 2nd cleavage sites which release C3f (not shown) and generates iC3(H_2O), and the 3rd cleavage to C3d,g and a C3c-like fragment (left). Alternatively, C3 is activated by the convertases which release C3a and exposes the TE in the formed C3b, which thereafter can get cleaved by factor I (right). C3a (α1) is indicated in red, linear in native C3 and titled in C3(H_2O) to symbolize that conformational changes occur after the thiol ester break, which lead to exposure of neoepitopes in C3a. C3d,g (α3) is shown in green and the β-chain and the 27 and 45 kDa fragments of C3c (α2 and α4) in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

forms which showed selectivity for zymosan and necrotic cells (Ferreira et al., 2010). In addition, in the characterization of the initial AP convertase and its stability in the fluid phase, the half-life of the formed complexes (both C3bBb and C3(H_2O)Bb) was shown to be increased 6 to 7 fold when Mg^{2+} was replaced by Ni^{2+} as the stabilizing cation (Fishelson et al., 1984). All these actions taken are natural in order to be able to characterize the convertase but makes the assessment of the C3b-like activity difficult.

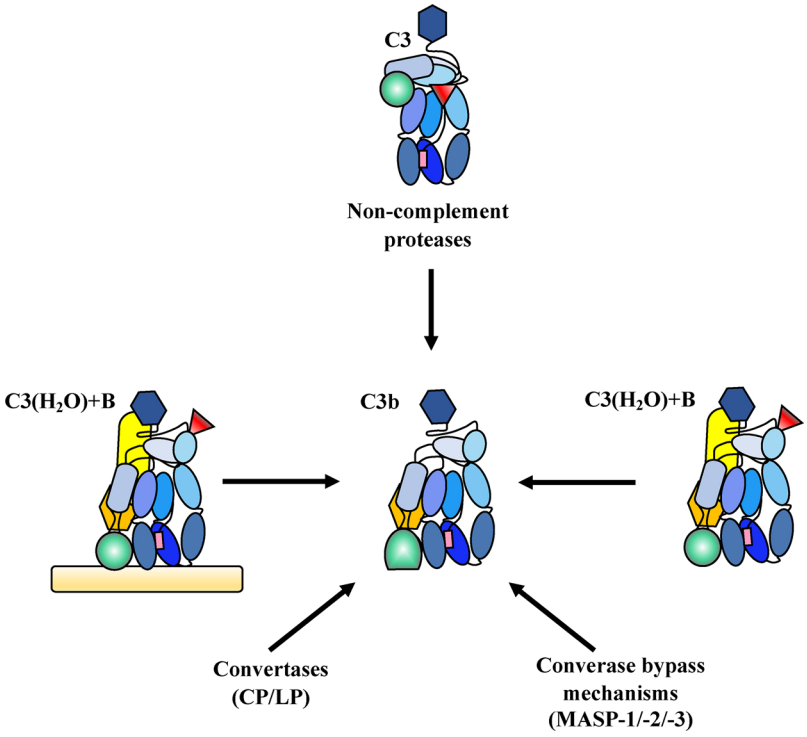

Fig. 3. Proposed mechanisms for the initiation of AP activation in human blood. The initial C3b which triggers AP activation can potentially be generated by proteases outside the complement system such as coagulation enzymes or renin (top) or by C3 convertases generated by the CP/LP (bottom left). In addition, specific activation of the AP by MASP-1 and possibly also MASP-2 has been described (bottom right). Furthermore, C3(H_2O) bound to surfaces (left) and possibly also in the fluid phase (right) is able to bind factor B (yellow) and generate functional AP convertases. The structures of C3 and C3(H_2O) are adapted from (Gros et al., 2008) with permission from the publisher. The binding of factor B (opposite to C3a in red) is as reported in (Chen et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The conclusion from these studies are that C3(H_2O) obtains "C3b-like" properties but is a much poorer partner in the AP convertase than C3b. From previous studies there is clear evidence that C3(H_2O) forms a complex with factor B but except for the original observations using C3Nef, purified properdin or Ni^{2+}, few examples exist that it actually forms an efficient active convertase (Bexborn et al., 2008; Fishelson et al., 1984; Isenman et al., 1981; Pangburn et al., 1981; Pangburn and Müller-Eberhard, 1980). However, in our hands, using fully converted C3, only minute convertase activity is obtained with methylamine treated C3 (without the presence of C3Nef, properdin or Ni^{2+}) compared with C3b; but the C3(H_2O) is cleaved by factors I and H. In the presence of factor I and factor H in the convertase mixture, no convertase activity at all can be detected, suggesting that this is a poor mechanism for generating C3b and initiating AP activation.

3.3. Activated coagulation factors and other proteases

A number of non-complement serine proteases such as elastase, chymotrypsin and trypsin are also able to cleave native C3 to C3a and C3b in addition to the C3 convertases (Fig. 3, top). Furthermore, activated factors of the coagulation and contact activation and fibrinolysis systems have been shown to produce anaphylactic activity by generating C3a. The serine proteases FXa, thrombin, FIXa, and FXIa (in decreasing order of potency), as well as kallikrein, and plasmin have all been observed to have this activity when studied using *in vitro* systems with purified proteases and complement components (Amara et al., 2010; Foley et al., 2016; Irmscher et al., 2018). Also, complement activation was triggered by FXa when it was added to serum at a relatively low concentration ($\approx 0.2\,\mu g/mL$). The activation resulted in C3a generation (i.e., increased anaphylactic activity) and decreased hemolytic activity. The complement activation could be blocked by clinically applied anticoagulants, e.g., fondaparinux (Amara et al., 2010). These studies show that activated coagulation factors have the capacity to activate complement in a complex serum milieu. However, it is very likely that such an activity would be lower in plasma since it contains intact native coagulation factors, which are the preferred substrates for coagulation proteases. Many experiments with purified components have been performed using concentrations of proteases close to the theoretical limit, i.e., if the total amount of each zymogen present in plasma had been converted to active enzymes. In addition, in these studies each of these proteases have been tested separately, so consequently, the question of possible synergistic effects has not been addressed (Amara et al., 2010; Foley et al., 2016). There are, however, several situations in a clinical setting in which these conditions may be present in a local micro-milieu or even systemically (i.e., total activation of all available zymogen molecules), for instance in disseminated intravascular coagulopathy, traumatic-hemorrhagic shock, sepsis or multiple organ failure, when all these systems are exhausted, and the intravascular protease inhibitors have been consumed e.g., (Choi et al., 2014; Lim et al., 2003). Furthermore, under such dysregulated circumstances, a small amount of C3 activation may be sufficient to initiate a full-blown inflammation as a result of amplification by the AP of complement, regardless of the nature of the activating protease(s).

Another set of serine proteases is the human tissue kallikrein family consisting of fifteen released proteases (KLK1-KLK15) which are synthesized in tissues mainly of epithelial origin, and which are distinct from plasma kallikrein (Prassas et al., 2015). Several of the KLKs (e.g., KLK5, KLK6 and KLK14) have been postulated to have a role in progression and outcome of several forms of cancer such as breast and skin carcinoma (Borgoño and Diamandis, 2004). Out of these, KLK14 has been reported to cleave C3 thereby generating functionally active C3a without downstream generation of C5a (Oikonomopoulou et al., 2013).

All of these mentioned proteases may contribute to the tick-over of C3 to C3a and C3b. The concept of cleavage of C3 and other complement proteins by non-complement proteases has been reviewed in (Huber-Lang et al., 2017).

3.4. Cleavage of C3 by renin

Of particular interest, the Karpman laboratory recently demonstrated that renin, a kidney-specific enzyme with blood pressure regulating properties, cleaved C3 into C3b and C3a at pg/mL concentrations, in a manner identical to that of the C3 convertases (Fig. 3, top). Cleavage was specifically blocked by the clinically relevant renin-inhibitor aliskiren (Békássy et al., 2018). Renin-mediated C3 cleavage and its inhibition by aliskiren also occurred in blood plasma, where renin can be detected at ng/mL levels. Of interest in this case is that C3 is able to activate the renal renin-angiotensin system (Zhou et al., 2013) in a murine model and that C3aR and C5aR double knockout (KO) mice decreased both systolic and diastolic blood pressure in response to Ang II in a T cell-dependent fashion compared with WT (wild type), single C3aR-deficient (C3aR-/-), or C5aR-deficient (C5aR-/-) mice (Chen et al., 2018). The link is even more intriguing when considering that C3 and C3a are clearly associated with hypertension and might reflect that C3 and its cleavage products is a component of the regulation of blood pressure.

4. Homeostasis and attack

In the following text we will discuss the mechanisms by which the AP is activated. We will mirror the activation concepts used for the coagulation system "Hemostasis and Thrombosis" to be applied on the complement system and exchanged for "Homeostasis and Attack". In this context, "Homeostasis" can be regarded as the consequence of continuous and sufficient down regulation ("braking/deceleration") of the positive feedback loop and "Attack" as the consequence of excessive and uncontrolled activation ("acceleration").

4.1. Homeostasis: the regulated positive AP feedback loop

The AP is regulated by several mechanisms. Factor I cleaves the α'-chain of C3b at two sites into the fragment iC3b: between Arg^{1281}-Ser^{1282} and Arg^{1289}-Ser^{1290} (Fig. 2), respectively, in the presence of a cofactor, e.g., factor H, complement receptor 1 (CR1), membrane cofactor protein (MCP), factor H related protein-1 (FHL-1) (Harrison and Lachmann, 1980; Medof et al., 1982). iC3b is unable to bind factor B (Nicol and Lachmann, 1973; Pangburn et al., 1977; Whaley and Thompson, 1978). In addition to being co-factors to factor I, factor H, DAF and CR1 also accelerate the dissociation of the alternative convertase (Nicholson-Weller et al., 1982; Whaley and Thompson, 1978). The only regulator of the AP that stabilizes the convertase is properdin (Fearon and Austen, 1975). Factor H and properdin assist the AP to distinguish between different activating target surfaces (Harboe et al., 2017; Pangburn and Müller-Eberhard, 1978; Spitzer et al., 2007)

Lack of or deficient regulation on complement target surfaces is linked to disease and are mediated by deficient expression (exposure) of regulators, mutations in the regulators that lead to low function or mutations in complement activators leading to gain-of-function.

Microorganisms are targets where regulators may be missing although e.g. bacteria, viruses etc, often express species-specific regulators or pulls down complement fluid phase regulators such as factor H and C4b binding protein from the host plasma (Zipfel et al., 2013). Lack of regulation may here facilitate AP activation, but many of these microorganisms also expose particularly carbohydrate structures that bind complement recognition molecules or antibodies that promote C3b deposition on the surface and therefore both dysregulated homeostasis and attack apply. The same thing can be said about biomaterial surfaces of e.g. hemodialysis devices, which in most cases are devoid of regulators on the material surfaces. The activation is in this case predominantly via the AP, but is in most cases preceded by the CP or LP both *in vitro* and *in vivo,* which abrogates the lag phase which is typical of the pure AP activation (Lhotta et al., 1998; Nilsson, 2001; Nilsson et al., 1993).

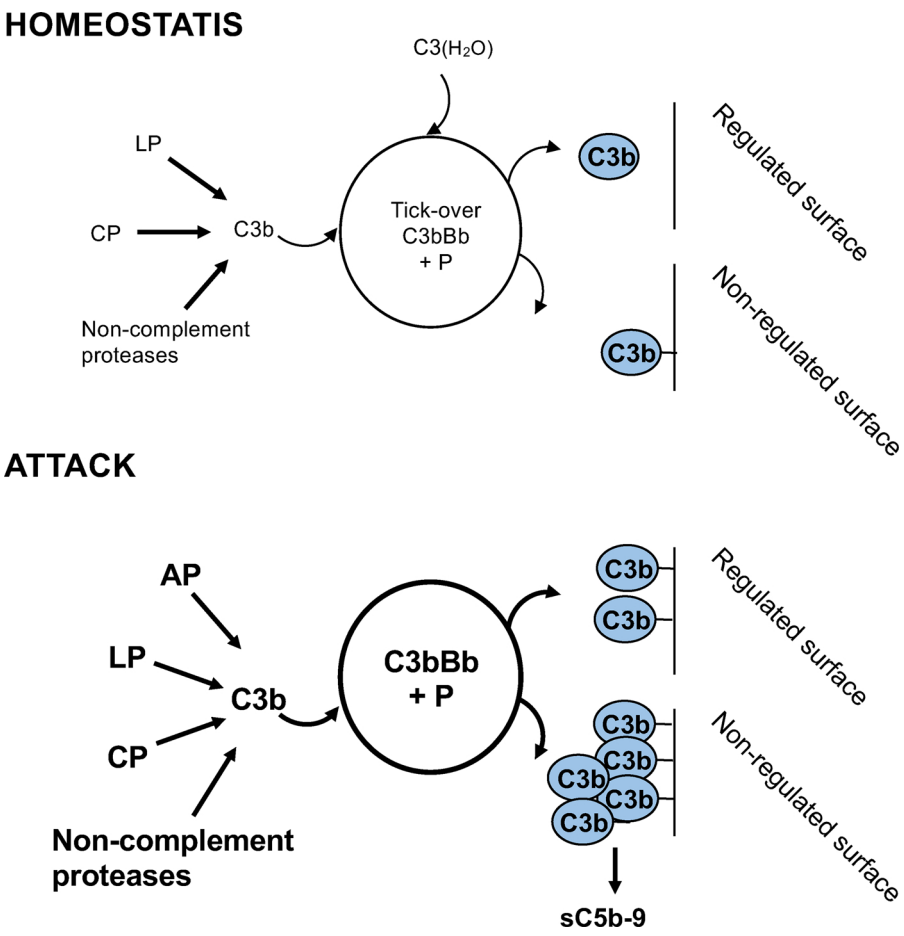

Fig. 4. Schematic overview of AP activation, amplification and regulation under conditions of Homeostasis and Attack. During homeostatic conditions (upper panel) low amounts of C3b are formed according to the tick-over theory in sufficient amounts to be able to interact with factor B and form a fluid-phase convertase. Activation may occur via the, LP, the CP, by non-complement proteases, or via C3(H_2O) dependent mechanisms. The availability of complement regulators (surface-bound or the fluid phase) determines to what extent the generated C3b is deposited on target surfaces. I.e., homeostasis can be regarded as the consequence of continuous and sufficient down regulation.
Under conditions of attack (lower panel) the LP, CP and non-complement proteases provide C3b molecules which when they have reached a threshold value fuels the AP leading to generation of vast amounts of C3b due to its potent amplification loop. C3b-deposition initiates inflammation both on surfaces and in the fluid phase. Given the massive output of C3b, there may also be activation on autologous cells surfaces because the density and efficacy of the available inhibitors is insufficient to control such substantial complement activation. i.e., attack can be regarded as the consequence of excessive and uncontrolled activation.

Age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobulinuria (PNH) are typical examples of rare diseases when the homeostasis is disturbed and the AP positive feedback loop is accelerated leading to damage and inflammation. Both aHUS and PNH are typically relapsing diseases where exacerbation may be due to periodically more generation of C3b, e.g., infections, that alters the balance in the AP homeostasis (Fig. 4).

4.2. Attack: the LP and the CP mediate recognition-restricted C3b deposits that feed into the positive AP feedback loop

The positive feedback nature of the AP creates an amplification loop which is the essence of the AP. In order to avoid a long lag phase before the activation takes off, a large amount of initial C3b molecules bound to the target surface is needed in order to speed up the activation. Without available C3b molecules, the lag phase can be very extended since the C3b generation is exponentially starting from one potential molecule (Schreiber et al., 1978). This is very well illustrated on a biomaterial surface both *in vitro* and *in vivo* where this lag phase may last up to 5–10 min (Lhotta et al., 1998; Nilsson, 2001; Nilsson et al., 1993). The CP and LP provide these C3b molecules and allow AP activation to start immediately, thereby adding specificity to the AP which is governed by the specificity of the CP and the LP (Fig. 3, lower left, Fig. 4). However, the vast majority of C3b molecules, particularly in inflammatory reactions, are generated by the AP convertases, even if complement activation is initiated by the CP or LP. This indicates that the AP in this case is an amplification mechanism and not an activation pathway (Harboe and Mollnes, 2008). In other words, a relatively large number of bound C3b is necessary in order to obtain an efficient activation of the AP.

4.3. Attack: Solid phase-bound C3(H_2O) forms initiating AP convertases leading to deposition of C3b

Previous studies show that C3 readily is adsorbed to different types of surfaces. We investigated the biological activity of C3 adsorbed to polystyrene in microtiter plates and found that native C3 was “activated” by the adsorption process (Andersson et al., 2002). We characterized the bound C3 by Quartz Crystal Microbalance with Dissipation (QCM-D), employing monoclonal antibodies (mAbs) directed against C3 neo-epitopes, indicating that the adsorbed C3 had acquired a C3b-like conformation (Nilsson et al., 1987) (Fig. 3, left, Fig. 4). The binding pattern of these antibodies was similar to that for adsorbed C3b. Adsorbed C3 was able to initiate the assembly of the AP convertase in a fashion similar to C3b, but unlike C3b or C3(H_2O) in the fluid phase or bound to platelet or platelet-derived microparticles, it was poorly regulated by factors I and H. These experiments revealed that adsorbed C3 can act as a trigger of the AP (Andersson et al., 2002) by altering the balance in the homeostasis and increase the amount of C3b that can initiate AP activation.

5. Specific activation of the AP by the LP

Recently, Gal et al. showed that inhibition of MASP-1 was able to inhibit the AP when activated by LPS (Paréj et al., 2018), demonstrating that the LP is directly involved in the activation of the AP. Other types of classical AP target surfaces, e.g. rabbit erythrocytes and zymosan were not affected. The mechanism that explains the MASP-1 dependence of the AP is still not known. The existence of an MBL-dependent C2 bypass mechanism for AP-mediated C3 activation was clearly demonstrated already by Selander et al. Their conclusion was that MASP-1 was likely to contribute, but was not required for C3 deposition in the

model used (Selander et al., 2006). Later studies suggested that this bypass mechanism also involved MASP-2 (Yaseen et al., 2017).

Further examples that LP is intermingled with the AP is that factor D, the protease that cleaves factor B in complex with C3b thus generating the AP convertase, exist in a zymogen form (pro-factor D) which is cleaved by the LP. The protease that drives this cleavage and generates active factor D, was for a long time unknown, but in 2011 the Fujita group demonstrated that MASP-3 KO mice lack AP activation (Iwaki et al., 2011). From these studies it was shown that MASP-3 was able to cleave pro-factor D into active factor D, but it was still unclear whether this was valid also in humans. Later studies by Gal et al. demonstrated that in humans, MASP-3, although not being the only protease, was the predominant protease that generated active factor D (Dobó et al., 2016). Taken together, from these results, it can be concluded that several links exist between the LP and AP involving the different MASPs (Fig. 3, lower right).

6. Other biological functions of C3(H_2O)

6.1. The half-life of C3 in plasma

The half-life of C3 in blood plasma is approximately 3 days (72 h). With a hydrolysis of the thioester at a rate of 0.4%/hr (Pangburn et al., 1981) and the fact that the C3(H_2O) is taken up by C3 receptor-bearing cells it is likely that the hydrolysis contributes substantially to the clearance of native C3.

6.2. Biological functions of bound C3(H_2O)

Native C3 and C3(H_2O) can bind to a surface by different mechanism. In biomaterial applications both, native C3 and C3(H_2O) can adsorb to the surface of a material or a gas bubble whereby the conformation of the molecule is altered (Fig. 5, bottom, left). Many of the neoepitopes exposed by biologically bound C3 fragment are then detected by neoepitope antibodies on the adsorbed proteins. C3(H_2O) can also bind to biological surfaces via complement receptors or via properdin and (Saggu et al., 2013). In all these instances the molecule acquired different biological functions (Fig. 5, right).

In addition to being able to act as a ligand for CR1 (CD35) (Nilsson Ekdahl et al., 1992), surface bound C3(H_2O) also binds to CR3 (CD11b). This was demonstrated using QCM-D where recombinant Mac-1 (CD11b/CD18) bound to sensors coated with C3(H_2O), but not to sensors coated with control proteins. Furthermore, CHO-cells expressing Mac-1 but not CHO-cells were shown to bind to C3(H_2O) coated onto polystyrene plates. In both experimental setups, interaction was seen between C3(H_2O) and CD11b which was inhibitied by specific mAbs (Hamad et al., 2015).

6.3. Liposome-bound C3(H_2O)

Liposomes are artificial phospholipid-bilayer particles which are used as drug delivery systems, as they can entrap hydrophilic or hydrophobic drugs in their aqueous core as well as insert hydrophobic drugs in their lipid bilayer (Czogalla et al., 2014). Liposomes in contact with blood induce complement activation by the CP (negatively charged) or the AP (positively charged) depending on their surface charge (Chonn et al., 1991; Moghimi et al., 2011). In contrast to the conventional proteolytic activation by convertases, we have demonstrated that neutral liposomes mediate a non-proteolytic activation of C3, leading to deposition of C3(H_2O) on the liposome surfaces (Fig. 5, right), as well as generation of complement activation products in the fluid phase (Klapper et al., 2014). These results are in line with those of Hourcade et al., who observed that purified native C3 when added to lipid-encapsulated nanoparticles acquired a “C3b-like” conformation, i.e., C3(H_2O), as demonstrated by its sensitivity to cleavage by factor I with factor H as co-factor (Pham et al., 2011).

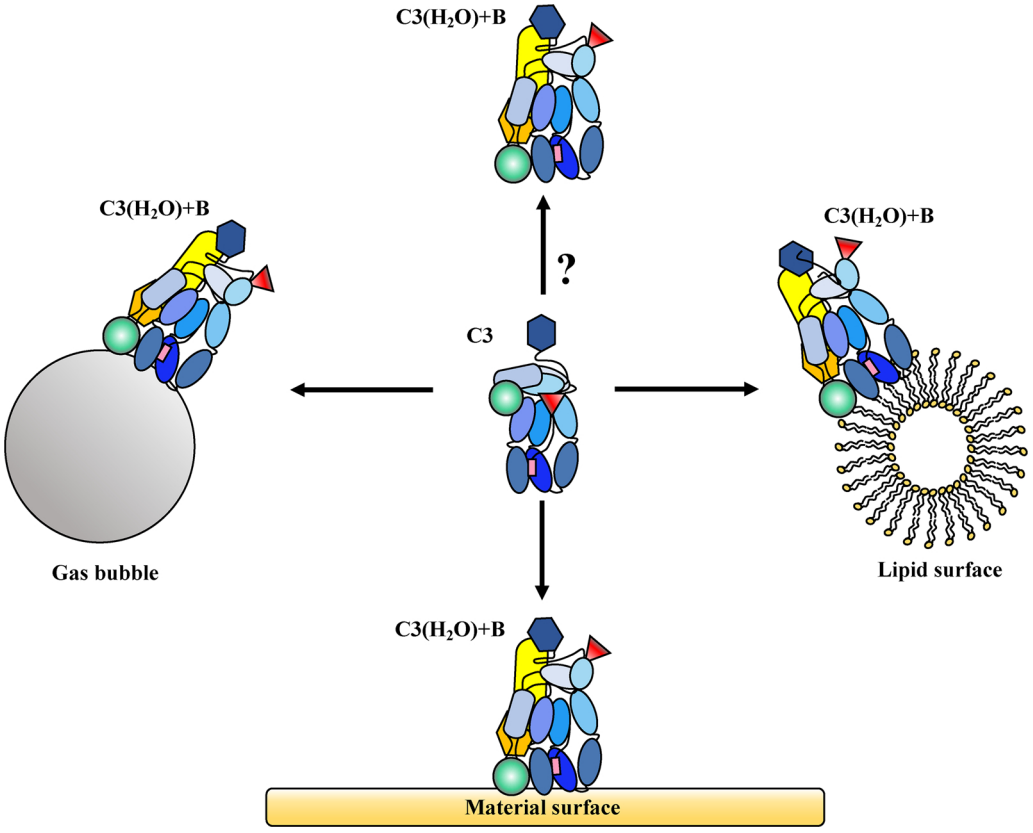

Fig. 5. Proposed mechanisms for the generation of C3(H_2O) in human blood. When native C3 (middle) comes in contact with the interface of gas bubbles it becomes conformationally changed to C3(H_2O); (left). Similar conformational changes occur when C3 is adsorbed to lipid surfaces such as therapeutic liposomes (right), to artificial surfaces such as in different biomaterials (bottom), or by spontaneous hydrolysis (top). In each case, the formed C3(H_2O) may bind factor B (yellow) which binds opposite to the C3a domain (red) as reported in (Chen et al., 2016). The bound factor B can in some cases get activated by factor D to form a C3 convertase complex, and the non-covalently bound surface adsorbed C3(H_2O)B, or C3(H_2O)Bb can be released into fluid phase (not shown). The figure is adapted from (Nilsson and Nilsson Ekdahl, 2012) and the structures of C3 and C3(H_2O) from (Gros et al., 2008), in both cases with permission from the publisher. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

6.4. Platelet-bound C3(H_2O)

In a series of publications, we have demonstrated that C3 binds to activated platelets but independently of complement activation. Under our experimental conditions: thrombin receptor agonist peptide (TRAP) activation of platelets in lepirudin anticoagulated platelet rich plasma, we could show that the binding of C3 to the surface was not inhibited by specific complement inhibitors (a blocking antibody to C1q or the C3 inhibitor compstatin, respectively) nor by the presence of chelators (Hamad et al., 2010). The nature of the bound C3 to the activated platelets was investigated by flow cytometry using two mAbs specific for C3c (C3-9) and C3a (4SD17.3) which do not bind to native C3 (see Fig. 2 for the location of the binding epitopes). Binding of C3-9 to activated platelets demonstrated that the bound C3 protein was an activated form of C3 while binding of 4SD17.3 demonstrated that this form of C3 contained the C3a moiety. Taken together, this analysis suggested that C3(H_2O) binds to activated platelets. This interpretation was confirmed by subsequent Western blot analysis which showed a fragment of 75–80 kDa that was detectable with anti-C3a, i.e., the N-terminal end of the factor I-cleaved α-chain of C3(H_2O), iC3(H_2O) (Hamad et al., 2010). Furthermore, microparticles derived from human serum showed an identical picture with factor I-cleaved iC3(H_2O) as did intact activated platelets (Hamad et al., 2015).

The C3(H_2O) enabled tethering between activated platelets and activated PMNs and monocytes. This interaction was blocked by anti-C3a mAbs and anti-CD11b mAbs, indicating that both, C3(H_2O) and C11b/CD18 are involved in this interplay. Furthermore, C3(H_2O)-bearing platelet-derived microparticles were also found to tether to activated PMNs and similar inhibition of the binding was seen using the same inhibitory mAbs as for platelets (Hamad et al., 2015).

These findings are in line with results from Saggu et al., who reported that physiological forms of properdin can bind to human platelets after activation and recruit C3(H_2O) to the surface (Saggu et al., 2013). The bound C3(H_2O) is acting together with properdin as a bridge in platelet-PMN complexes. Taken together these results suggest alternative ways for activated platelets to contribute to localized inflammation.

6.5. The uptake of C3(H_2O) by fibroblasts and leukocytes

The first report of ingestion of C3(H_2O) by intact cells appeared 2 decades ago when Meilinger and collaborators demonstrated that mouse fibroblasts in culture were able to take up C3(H_2O), but not native C3, via the low density lipoprotein receptor-related protein/α_2 macroglobulin (LRP/α_2M) receptor which therefore was postulated to participate in C3 metabolism (Meilinger et al., 1999).

Many types of leukocytes, most markedly activated native B-cells, are able to take up C3 in the form of C3(H_2O) from serum or a solution of purified C3 *in vitro* (Liszewski et al., 2017) (Elvington et al., 2017). C3 has been identified in freshly isolated human primary B-cells but not in the corresponding cell lines (Farage cells). However, when the Farage cells were exposed to serum or purified C3, the cells were loaded with the protein. Interestingly the fraction of C3, that was preferred for uptake was also in this case C3(H_2O), but despite intense studies the binding receptor(s) remain(s) to be identified (Elvington et al., 2017). The ingested C3 is proposed to be a part of the turn-over of the intracellular complement system (Arbore et al., 2017). Further studies showed that in addition to B-cells also T-cells and other cell types also take up C3(H_2O) (Liszewski et al., 2017).

Whether or not the ingested C3(H_2O) is cleaved to biologically active fragments (keeping in mind that cleavage of C3(H_2O) does not readily give rise to C3a) is presumably a consequence of the available non-complement and complement proteases and co-factors in combination with the pH in the subcellular compartments where the C3(H_2O) it is internalized (lysosomes or cytoplasm). E.g., the pH optimum for factor I cleavage of C3b when factor H is the cofactor is low (< 5.5) but around 7.5 (i.e., similar to that of other serine proteases) when CR1 is the cofactor (Sim and Sim, 1983; Tsiftsoglou and Sim, 2004). To our knowledge, no corresponding studies regarding pH optimum for cleavage of C3 or C3(H_2O) have been made, neither for factor I together with other cofactors, e.g., MCP, nor for other non-complement intracellular proteases.

7. Quantification of fluid-phase C3(H_2O)

The internal thiol ester of C3 is susceptible to nucleophilic attack or hydrolysis resulting in C3(H_2O) where the α-chain still contains the C3a moiety. Therefore, immunochemical quantification of fluid phase C3(H_2O) can be performed by sandwich ELISAs using combinations of a mAb anti-neo-C3 for capture and mono- or poly-clonal anti-C3 for detection (see Fig. 2 for the location of the binding epitopes). Such an assay was developed in our laboratory in the 1990s using a neo-epitope mAb anti-C3a (4SD17.3) for capture and polyclonal anti-C3d or anti-C3c for detection (Nilsson Ekdahl et al., 1992). We have used this assay to demonstrate that C3 becomes conformationally changed and activated in human blood and plasma *in vitro* on bubbles of gas of different composition (O_2, N_2, and air) and during oxygenation of human blood during a bench top experiment with a bubble oxygenator (Fig. 5, left). It was also demonstrated that the generated C3(H_2O) bound to washed human erythrocytes, suggesting affinity for CR1 since this is the only known receptor for C3 fragments on RBCs (Nilsson Ekdahl et al., 1992). Finally, we confirmed the presence of C3(H_2O) in samples collected from patients during cardiopulmonary bypass, in high amounts using bubble and in lower amounts using membrane oxygenators (Pekna et al., 1993).

This assay detects intact C3(H_2O) as well as the factor I generated cleavage product iC3(H_2O) in which the α-chain has been split into fragments of 75–80 kDa and 45 kDa (see above). In this context it is important to note that if polyclonal anti-C3c is used for detection instead of anti-C3d, the assay will also detect the C3a-containing form of C3c (Fig. 2).

Confirmatory analyses can be made by Western blotting analysis using epitope mapped mAbs or polyclonal antisera. This technique will not provide information on the conformation of the C3 population in solution due to the denaturation which takes place during the SDS-PAGE step. However, it will show whether the C3 was cleaved by factor I prior to electrophoresis, which is an indirect proof of the presence of C3(H_2O) since native C3 in the fluid phase is resistant to factor I. This was the case of the C3 population detected in samples collected during cardiopulmonary bypass, confirming that C3(H_2O) indeed was generated in real life in these patients (Pekna et al., 1993).

In addition, we have detected increased levels of non-proteolytically activated C3 in patients with primary biliary cirrhosis; PBC (Nilsson et al., 1986) and in patients with hepatic encephalopathy and elevated levels of ammonia as compared to healthy controls (Ekdahl et al., unpublished data). At least in the case of PBC, the C3(H_2O) was associated to high molecular weight lipid-like material. Since C3(H_2O), both when isolated from plasma from PBC patients and after *in vitro* generation by gas bubbles, has been shown to bind to bind to CR1 (Nilsson Ekdahl et al., 1992; Nilsson et al., 1986) it may be expected to impair the handing of immune complexes, as is frequently seen in PBC (Nilsson et al., 1986). Alternatively, it could be speculated that C3(H_2O) generated *in vivo* may be ingested by leukocytes as mentioned above, thereby topping up their intracellular pool of C3.

An assay for detection of C3(H_2O) with a similar rationale, i.e., using a neo-C3 mAb for capture, in this case a C3b/iC3b mAb (clone 3E7) and a polyclonal anti-human C3a antiserum for detection was recently published (Elvington et al., 2019). These authors also report enhanced levels of C3(H_2O) in serum samples from patients with systemic lupus erythematosus and rheumatoid arthritis compared to healthy controls.

For analytical or preparative purposes, C3 and C3(H_2O) can be

separated by FPLC on a MonoS column using a gradient of NaCl from 80 to 700 mM in 50 mM acetate buffer, pH 5.2 (Hack et al., 1990).

8. Conclusion

In the alternative pathway (AP) an amplification loop is formed, which is strictly controlled by various fluid-phase and cell-bound regulators leading to a state of homeostasis with a slow-rate formation of AP convertases (C3bBb) able to cleave C3 to C3b and C3a, and continuously deposit C3b molecules on target surfaces. Since the first characterization of the "C3b-like" C3(H_2O), it has been described as essential for AP activation, because it explains how the initial C3b molecules can be generated. Despite detailed regulation by, e.g., factor H and properdin on various surfaces, the AP has also been considered to be an unspecific pathway.

During complement attack in pathological conditions and inflammation, large amounts of C3b are formed by the classical/lectin pathway (CP/LP) convertases. After the discovery of the LP´s recognition molecules and its tight interaction with the AP, it is increasingly likely that the AP acts *in vivo* as a powerful amplification mechanism of complement activation that is triggered by previously generated C3b molecules initiated by the binding of specific recognition molecules and immunoglobulins to the target surface.

Also, in many pathological conditions caused by a dysregulated AP amplification loop such as in paroxysmal nocturnal hemoglobulinuria (PNH) and atypical hemolytic uremic syndrome (aHUS), C3b is available due to minute LP and CP activation and/or several other mechanisms. E.g., contact surface activation and cleavage by non-complement proteases, such as renin, which makes, at least *in vivo*, the hydrolysis of the thioester of C3 into C3(H_2O) less necessary to explain how the tick-over of C3b molecules is brought about during the initial AP convertase formation.

Therefore, C3(H_2O) generation *in vivo* may be less important for AP activation during specific *attack* or dysregulated *homeostasis*, but C3(H_2O) may be an important ligand for C3 receptors in cell-cell interactions and a source of C3 for the intracellular complement reservoir.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by grants 2016-2075-5.1 and 2016-04519 from the Swedish Research Council (VR), by faculty grants from the Linnaeus University, and from Deutsche Forschungsgemeinschaft (DFG) grant CRC1149A01.

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