Looking back on the alternative complement pathway

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Introduction

It is not the intention of this paper to review in great detail all that is known of the "alternative complement pathway". There have been several reviews in recent years (Bexborn *et al* 2008; Lachmann 2009; Nilsson and Nilsson-Ekdahl 2012; and Harrison 2017;) the last of which in particular goes into the subject in great detail. However, there does appear to remain a degree of misunderstanding of some aspects of this pathway particularly in the role of the "tickover" which is still frequently shown in diagrams of the alternative pathway as its activating event – which, as is discussed below, it is not. The intention of this paper is to look again at what the alternative pathway is and what it does and to suggest that it should be regarded in a different light from the classical and lectin pathways, serving in large part as an amplification mechanism for all ways of activating the complement system – a conclusion similarly reached by Harrison (2017).

History

What became known as the alternative complement pathway originated with the work of the Pillemer laboratory when the first paper on "the properdin system and immunity" was published in 1954. This work gave rise to a series of some fourteen papers followed by immense controversy which are comprehensively reviewed by Lepow (1980). It is perhaps just worthwhile with hindsight to look at this early work in outline again. The Pillemer group used zymosan, a preparation of yeast cell walls that is pure carbohydrate, as a complement activator. It was already then known that treating human serum at 37^{o}C with zymosan depleted it of what was then called "C'3", which then described all the components needed after C1, 4 and 2 to produce lysis. Treating guinea pig serum with zymosan does remove all C3 (in its contemporary sense) so that the resulting reagent, known as "R3", can be used to generate EAC142 when added, in the presence of calcium, to antibody-coated erythrocytes. This is not the case with human serum where the R3 reagent seems to exhaust Factor B rather than C3 and the human "R3" does contain appreciable amounts of C3. When used to treat antibody-coated erythrocytes in the presence of calcium and magnesium human R3 gives rise to the intermediate EAC1423bi. None of this this was known in the 1950s since C3, the first complement to be isolated as a protein, was not described until 1960 (Müller-Eberhard and Nilsson 1960).

The observation that formed the basis of the work on properdin was that when zymosan was incubated with human serum at 17ºC, it removed a component (to which the name properdin was given) which was necessary for zymosan to produce an R3 reagent at 37ºC. Properdin was subsequently purified by Pensky *et al* in 1968 by which time it had also been discovered that there were other components required for the fixation of properdin to zymosan to take place. These were recognised as being similar to the classical complement components C1, C4 and C2. Looking back at these experiments with the hindsight of over sixty years, it does seem to be the case that at 17ºC there is a reaction that allows covalent binding of C3b to zymosan where it may bind some Factor B (the C2-like component of the properdin pathway), and that this then allows the binding of properdin to C3b or C3b(B). The exact nature of the reaction that allows the C3 fixation is still not wholly clear. Pillemer and his group used a resin to deplete cations and showed that they needed to restore only magnesium, rather than calcium, in order for this reaction to work. If this resin removed all calcium, then one can exclude the classical pathway as being involved. Otherwise it certainly would be activated, since human serum contains antibodies to the many carbohydrate determinants that are found on zymosan. Total absence of calcium would also exclude involvement of much of the lectin pathway (which was quite unknown at that time) since the C-type lectins - mannose binding lectin, and the collectins - also require calcium for their activity. On the other hand, it is now known that ficolins are another group of proteins that can activate the lectin pathway. These are not C-type lectins but use fibrinogen-type recognition domains which, generally, do not require calcium for binding their ligands (Garlatti *et al* 2010). It is therefore quite plausible that this reaction at 17ºC which allows the covalent fixation of C3 onto zymosan is mediated by the lectin pathway using ficolins. Furthermore, the discovery by Yaseen *et al* (2017) that MASP-2, the principal enzyme involved in lectin pathway activity going via C4 and C2, can itself produce sufficient cleavage of C3 to activate the alternative pathway, provided another route by which lectin pathway activation can interact directly with the alternative pathway. It is quite likely, therefore, that what Pillemer was observing was due, in part at least, to lectin pathway activation. It is also possible that it is the “protected surface” property of zymosan – its capacity of allowing bound C3b to bind Factor B in preference to Factor H – that allows some C3 fixation to occur. However, the C3b amplification loop does not work well at 17ºC and in order to get appreciable

C3 fixation by such a mechanism one would anticipate the need for a much higher temperature.

What generated controversy was, largely, the claims that properdin was a major player in innate immunity to viruses, bacteria and tumours. There is some dispute whether Pillemer made these claims in quite the way it was reported in the popular press at the time (see the discussion by Colten quoted in Lachmann (2006)but these claims certainly raised the profile of the properdin system and also, probably, encouraged the scepticism. The major sceptic was Robert Nelson who in 1958 published “an alternative mechanism for the properdin system” having previously presented his criticisms at meetings. Nelson’s view was that basically the properdin experiments were simply demonstrating the activity of the classical pathway that involved antibodies and classical pathway components. His experiments were, however, done in large part on guinea pig serum which, as already mentioned, is not an entirely good analogue for human serum with regard to reactions with zymosan and certainly what Nelson described would have required the presence of calcium. In retrospect, therefore, his criticisms do not look as potent as they were considered at the time.

Properdin was shown to act as a stabiliser of the alternative pathway convertase C3bBb (Fearon and Austen 1975) and as such has an important physiological role. Properdin deficiency is associated with meningococcal infections, as are so many other complement deficiencies. Moreover, it is now established that properdin, as it occurs in plasma, reacts only with C3b (Harboe *et al* 2017) in spite of more recent claims (Hourcade *et al* 2006; Kemper *et al* 2010) that properdin may act as a recognition molecule and therefore could act as an initiator of the complement alternative pathway along the lines originally postulated by Pillemer. However, larger polymers of properdin do have some capacity of this type. These polymers can be found in properdin purified from plasma where they are an artefact, as was first shown by Farries *et al* (1987). However, it has more recently been shown that properdin made by recombinant techniques (Ali *et al* 2014) has similar properties to the artefactual properdin polymers and this recombinant properdin has been shown to be a powerful stimulant of complement activity by stabilising the C3 convertase. Whether properdin found at extravascular sites in vivo, possibly made by

polymorphs, may contain or comprise these larger polymers is unknown and it is therefore still unclear whether these larger properdin polymers can occur *in vivo*.

Evolutionary History

In contrast to its discovery history, in evolutionarily terms the alternative pathway can be considered to form the oldest part of the complement system (see Lachmann 2009 for a more detailed account). A C3 like molecule can be found in insects and a Factor B-like protein in echinoderms. In these invertebrates the invading micro-organisms presumably supplied the enzymes needed to cleave C3 and Factor B. In vertebrates with a pumped circulation which contains a wide variety of protease inhibitors the remainder of the alternative pathway evolved (see Figure). Interestingly, echinoderms were also shown to be able to activate the C3 like molecule through analogues of lectin pathway components (Fujita, 2002) indicating that a form of invertebrate lectin pathway by far antedates the classical pathway activation route. The appearance of the latter somewhat paralleled the evolutionary rise of antibodies, the most potent inducers of classical pathway activation.The classical pathway seems to have developed in vertebrates largely by gene duplication to provide a feed-in to the alternative pathway particularly from the humoral adaptive immune system; and the vertebrate lectin pathway to allow activation of the classical pathway using lectins recognising pathogen associated carbohydrate determinants and thus providing a further feed-in from the innate immune system.

The renaissance of the Alternative Pathway

The renaissance of the alternative pathway following the controversies of the 1950s and early ‘60s came from a quite different direction which was the demonstration that there were various ways of activating complement that did not involve the early classical pathway components. Gewurz *et al* (1968) showed that lipopolysaccharides could consume what had been known as C’3 - which by that time was known to be all the components from C3 to C9 - without consuming C1 and C4. It was shown that precipitates made with guinea pig IgG1 antibodies (Sandberg *et al* 1970) or with rabbit Fab’2 (Reid 1971) had similar properties. In 1971, Frank *et al* showed that C4-deficient guinea pig complement was able to be activated using suitable activators. These findings made it amply clear that there was a mechanism

of activating the complement system that did not require C1, C4 and C2 and this renewed the study of what other components were involved in this pathway. It had already been found that these showed analogy to the classical pathway components. There was a C2-like (heat labile) protein which was demonstrated as a novel protein by Boenisch and Alper 1970 and who named it glycine-rich beta glycoprotein (GBG). A similar protein was described by Götze and Müller-Eberhard in 1971 and was called by them C3 proactivator and again by Goodkofsky and Lepow in 1972, from what had been the Pillemer laboratory, who called it Factor B by analogy with the original Pillemer nomenclature and this name was finally adopted. There was also a C4-like (hydrazine sensitive) protein which was known as properdin Factor A and this was demonstrated by Müller-Eberhard and Götze in 1972 to be C3 itself. A C1-like component was isolated by Alper and Rosen (1971) who called it glycine-rich beta glycoproteinase (GBGase) and the following year by Müller-Eberhard and Götze (1972) who called it C3PA convertase. This protein was subsequently called Factor D.

In the early 1970s these laboratories were of the opinion that this alternative pathway would have an initiation rather like the classical pathway and it was speculated that this would start, by analogy with the classical pathway, with the activation of Factor D from a precursor form (Fearon *et al* 1979). The Müller-Eberhard laboratory (Vallota *et al* 1974; Schreiber *et al* 1976a and b) believed that the initiation event involved a novel initiating factor which was a naturally occurring analogue of C3 nephritic factors, which had first been described by Spitzer *et al* (1969). However, neither of these hypotheses turned out to be correct. There is a zymogen form of Factor D but normal serum always contains active Factor D and the activation of the zymogen probably occurs largely extra-vascularly near the site of synthesis of Factor D, which is largely by adipocytes. It has recently been demonstrated that the enzyme that is largely concerned in this activation is MASP-3 (Dobó *et al* 2016), one of the enzymes of the lectin pathway, and the only one that is resistant to inhibition by C1 inhibitor (Zundel *et al* 2004) and therefore has a prolonged active half-life *in vivo*. The initiating factor turned out not to exist at all. Nephritic factors, contrary to the vigorous claims of the “La Jolla group” (i.e. Müller-Eberhard and co-workers), are indeed immunoglobulins and they are a curious set of autoantibodies to the alternative pathway C3 convertase which cause major activation of the complement

system. It is still unknown what causes them to be formed. They are, however, IgG, as first suggested by Thompson (1972), who identified them particularly with IgG3, and this was subsequently proven by showing that they pass the placenta (Davis *et al* 1977) and by Scott *et al* (1978) and Daha and van Es (1979) who showed that Fab and Fab'2 fragments of these antibodies retained their biological activity. Scott *et al* (1981) further showed that these antibodies were unusual in that they are rather larger than normal IgG; that this increase in size is due to increased glycosylation; and that removal of the carbohydrate with mild periodate treatment causes them to lose their biological activity. These "original" nephritic factors will, when purified as IgG and added to normal human serum, cause conversion of the C3 in the serum to iC3b and subsequently to C3dg and C3c. This reflects the findings in patients with these nephritic factors who have C3dg (known earlier as alpha 2D globulin) present in their plasma. These effects result from their property of powerfully stabilising the alternative pathway C3 convertase (Daha *et al* 1976). In more recent years, a new definition for nephritic factors was adopted which classifies as nephritic factors all antibodies that show some stabilisation of the alternative pathway convertase. The great majority of these do not convert C3 in normal human serum and their biological significance is less secure. One always has to bear in mind that antibodies to C3 breakdown products occur in response to complement fixation of all kinds and these antibodies are known as immunoconglutinins (Coombs *et al* 1961) so one must always be certain that such antibodies are the cause of the complement activation rather than its result.

The Alternative Pathway as a purely rate governed reaction

The recognition that the alternative pathway was quite different from the classical pathway – and subsequently from the lectin pathway as well - in having no initiating event at all again came from quite different studies. In 1967, Alper and his colleagues in Boston first studied a patient who had powerful continuous complement activation in vivo with conversion of C3 to C3b and of Factor B to Bb (Alper *et al* 1970**)**. It was at that time quite unknown what was the matter with him and the possibility of his abnormality being Factor I deficiency was discussed when Chester Alper and I met at the 17th Protides of the Biological Fluids meeting in 1969, Factor I having been discovered in 1966 as an enzymatic component of plasma that was required to convert C3b (which had been found not to react with bovine

conglutinin) to another C3 breakdown product, C3bi, that would do so (Lachmann and Müller-Eberhard 1968). The Boston patient, TJ, did turn out to be Factor I deficient and this was the cause of the continuous activation of his alternative pathway. It was subsequently shown (Nicol and Lachmann 1973) that exactly the same situation could be produced *in vitro* by the depletion of Factor I from normal human serum using purified Fab2' antibodies. When such depleted serum is warmed up, there is immediate and virtually complete conversion of C3 to C3b. From these observations came the realisation that the alternative pathway is a rate-governed reaction where two competing pathways act upon C3b. One, subsequently found to involve Factor H as well as Factor I (Whaley and Ruddy 1976), leads to the breakdown of C3b to iC3b and destroys the possibility of feedback, whereas the other is the C3b feedback cycle where C3b combines with Factor B and is acted upon by Factor D and gives rise to C3bBb, the alternative pathway C3 convertase (see Figure 1).

The C3-tickover

It was apparent on looking at this model that there had to be a mechanism to provide a miniscule amount of C3b, or some analogous protein, present at all times so that simply removing Factor I (or Factor H) could activate the pathway. This was given the name of the "C3 tickover" (Lachmann and Nicol 1974; Lachmann and Halbwachs 1975) by analogy with a car engine that has to be ticking over before pressing the accelerator causes the car to move. At the time, it was thought that small amounts of C3 conversion to C3b by a variety of enzymes, including other complement enzymes, would be the cause.

The whole idea was greatly resisted by the La Jolla group when it was first presented to the Coronado Complement workshop in 1973 and they continued to publish about an initiating factor for some years thereafter. However, in 1981 Pangburn *et al* suggested, without actually mentioning the tickover hypothesis, that the initial C3 product might be a spontaneous hydrolysis product breaking the internal thioester bond and that this product, iC3, was capable of reacting in both the breakdown and amplification loops. This is an attractive and plausible idea though very difficult to prove. The essence of the tickover is that there are tiny amounts of either C3b or iC3 present at all times which do not themselves cause complement activation and

presumably are rapidly converted to iC3b and iC3i; and which can allow complement activation in the absence of other complement pathway activity

The Protected Surface

The “protected surface” phenomenon described by Fearon and Austen (1977) is certainly the essential mechanism for giving rise to alternative pathway activation in the absence of the activity of the other complement pathways. This term describes the phenomenon whereby C3b bound on certain surfaces is relatively resistant to binding Factor H while continuing to bind Factor B normally thereby promoting the C3b amplification loop. These protected surfaces were typically carbohydrates lacking sialic acid (Kazatchkine *et al* 1979) but these are by no means the only products to do this. Zymosan, as used by Pillemer and everybody else ever since, is a canonical protected surface as is particulate inulin and as is bacterial lipopolysaccharide of the smooth variety. In the case of inulin, it is of particular interest that soluble inulin does not have a protected surface effect suggesting that it does need to be an insoluble surface. Smooth lipopolysaccharides containing carbohydrates may well be the most important in vivo activator by this mechanism and Gram negative infections acting in this way may be of considerable importance in a number of diseases. The “rough” endotoxin - Lipid A - binds C1q and activates the classical pathway (Lachmann and Nicol 1974) so that endotoxins may recruit the alternative pathway by two complementary mechanisms.

There are other protected surfaces that are important during complement activation. One of these is IgG. C3b, or presumably iC3, bound to IgG is protected as shown by Sissons *et al* (1979) and by Reiter and Fishelson (1989). This is important in allowing the classical pathway to feed efficiently into the C3b feedback loop. Furthermore, as shown by Meri and Pangburn (1990) in an important but seldom quoted paper, C3 bound covalently to the classical pathway convertase, C4b2b, is also protected and this enables it efficiently to give rise to a C5 convertase as well as potentially stimulating feedback. This property seems not to be shared by C3b bound to the alternative pathway convertase, C3bBb, and this would explain the greatly increased lytic efficiency of the classical pathway over the alternative pathway. No other explanation for this phenomenon had ever really been adduced.

That it is necessary for C3 to be bound onto an insoluble surface for the activation of the alternative pathway (Nilsson and Nilsson Ekdahl 2012) is true for activation via the protected surface but is not necessary for activation by the depletion of Factor I or Factor H or the addition of nephritic factors to serum *in vitro* all of which can activate the C3 feedback loop to exhaustion entirely in the fluid phase.

Factor H deficiency produces a somewhat similar phenotype to Factor I deficiency although other Factor I co-factors do allow the production of iC3b from C3b. This has important consequences because the reaction of iC3b with CR3 on neutrophils is an essential mechanism for complement-mediated immunopathology. This occurs in Factor H deficiency but not in Factor I deficiency (Rose *et al* 2008).

It therefore is probably accurate to regard the C3b amplification and feedback loops as an amplification mechanism for all forms of complement activation, be it by the classical pathway or by the lectin pathway or by the presence of nephritic factors, and that it is only the protected surface phenomenon which gives it status as an autonomous pathway of complement activation *in-vivo*. It is the protected surface rather than the tickover which should be shown as “initiating” activation by the alternative pathway.
In vivo there are likely to be many situations where the recruitment of the amplification loop via the classical pathway or the lectin pathway are as important.

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