PATTERN OF DEGRADATION OF HUMAN COMPLEMENT FRAGMENT, C3b

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Received 7 July 1981

1. Introduction

The complement system is a multi-enzyme cascade found in blood plasma and is a major defense system against invasion by foreign material [1]. Complement can be activated in plasma by two routes – the classical pathway, consisting of components C1, C4 and C2 and the alternative pathway which involves serine proteases B and $\bar{D}$. Both pathways result in activation of C3, the third component of complement which is the most abundant complement protein, present at 1.3 mg/ml in plasma, Clinically, C3 depletion and the appearance of serologically defined C3 degradation fragments are used as measures of complement activation.

C3 is synthesised as a single polypeptide chain precursor of ~200 000 M_r which is subsequently cleaved into the two polypeptide chains (α, 116 000 M_r; β, 70 000 M_r) of plasma C3 [2]. During complement activation, C3 is cleaved at a bond close to the amino-terminus of the α-chain forming two fragments, C3a (8000 M_r) and C3b (178 000 M_r) (see fig.4). C3b has the transient ability to bind covalently through an ester or amide bond to nearby hydroxyl or amino groups [3].

The complement protein C4 is similar to C3 in its structure and properties and is cleaved into C4a and C4b fragments when complement is activated via the classical pathway. C3b and C4b, once formed, are degraded by a specific protease (C3b/C4b inactivator) [1] and in each case C3b/C4b INA acts only in the presence of a specific glycoprotein cofactor-β_1H for C3b [4] and C4b-binding protein (C4bp) for C4b [5]. C4bp has also been reported to act as cofactor for C3b cleavage by C3b/C4b INA [6,7]. The α'-chain of C4b is cleaved in two places by C3b/C4b INA [8,9]. However, there is controversy as to whether the α'-chain of C3b is cleaved at a single peptide bond [6,7,10–14] or in two places [15] by C3b/C4b INA. The product of C3b degradation by β_1H and C3b/C4b INA is called C3bi. C3bi is itself broken down by unidentified serum proteases to final products C3c (140 000 M_r) and C3d (30 000 M_r). (see summary fig.4).

We present evidence which shows that C3b, like C4b, is cleaved in two places by C3b/C4b INA. The double cleavage pattern occurs in serum and also when C3b is incubated with isolated β_1H and C3b/C4b INA. The second of the two cleavages can be preferentially inhibited at low pH. The product formed by these two cleavages, C3bi, is a relatively stable species in serum, in that it is formed rapidly but its subsequent degradation is slow.

2. Materials and methods

2.1. *Proteins and sera*

C3 and C3b were prepared and radio-iodinated using Iodogen (Pierce and Warriner, Chester) as in [16]. β_1H, C3b/C4b INA and C4bp were purified according to [17], [11] and [9], respectively. Isolated C3b/C4b INA was found to lose activity on prolonged storage at 4°C, although no change in polypeptide chain structure was observed. Haemolytically inactive C3 was produced by incubation of native, isolated C3

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; MES, 4-morpholino-ethanesulphonic acid; M_r, relative molecular mass; VBS^{2+}, 2.5 mM sodium barbitone–HCl (pH 7.5) containing 145 mM NaCl, 0.15 mM $CaCl_2$ and 0.3 mM $MgCl_2$

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Published by Elsevier/North-Holland Biomedical Press
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in 100 mM hydrazine (pH 9.0) for 1 h at 37°C.

Human sera deficient in specific proteins were prepared by passage of pooled human serum through Sepharose 4B columns to which were bound monospecific mouse or rabbit antibodies to the appropriate protein. These affinity columns, prepared as in [18], were run in 250 mM NaCl, 50 mM potassium phosphate, 5 mM EDTA (pH 7.5) at 4°C to avoid complement activation.

2.2. *C3b Degradation*

In serum: Samples (0.5–15.0 μg) of ^{125}I-labelled C3, ^{125}I-labelled hydrazine-inactivated C3 or ^{125}I-labelled C3b (spec. act. $1.2 \times 10^4 - 1.2 \times 10^6$ cpm/μg) were added to 2.5–10% (v/v) serum in a total volume of 100 μl of VBS^{2+}. Where indicated, β_1H was added to a final concentration of 40 μg/ml, C4bp was added to a final concentration of 100 μg/ml and C3b/C4b INA was added to a final concentration of 4.5 μg/ml. These concentrations correspond approximately to physiological levels of β_1H, C4bp and C3b/C4b INA in 10% (v/v) human serum.

In purified system: In a total volume of 100 μl 20 mM sodium phosphate, 5 mM EDTA (pH 6.5) samples (0.5–45 μg) of radio-iodinated C3, C3b or hydrazine-inactivated C3 (spec. act. 1.2×10^4–1.2×10^6 cpm/μg) were incubated with 10–40 μg β_1H or C4bp and 0.15–0.2 μg C3b/C4b INA. In studies of the effect of pH, all proteins were dialysed into 150 mM NaCl and incubated in a composite buffer of 25 mM MES, 25 mM HEPES, 25 mM Tris and 25 mM glycine adjusted to the desired pH at 37°C, as in [19].

In both purified and serum systems, where mentioned in the text, soya bean trypsin inhibitor (SBTI) was added to a final concentration of 100 μg/ml and di-isopropyl fluorophosphate (DFP) was added to a final concentration of 10 mM. Reaction mixtures were incubated at 37°C and samples (10 μl) were withdrawn and mixed with 10 μl 200 mM sodium phosphate, 8 M urea, 2% (w/v) SDS (pH 7.0) at various times to stop the reaction.

SDS–PAGE of reduced and alkylated samples were performed as in [20]. Autoradiography of the stained, dried gels was used to observe the degradation pattern of the radio-labelled proteins. Quantitation of degradation of radiolabelled proteins was determined by counting radioactivity in 1 mm strips cut from individual gel tracks (LKB Wallac 1270 Rackgamma counter). In systems using isolated proteins, the rate of degradation was also calculated from scanning the intensity at 540 nm of protein bands stained with Coomassie blue, using a Gelman DCD 16 densitometer.

3. Results

3.1. *Cleavage of C3b with purified β_1H and C3b/C4b INA*

When ^{125}I-labelled C3b is incubated with C3b/C4b INA and β_1H, the α'-chain of C3b is degraded (fig.1a). In parallel with the decrease in intensity of the α'-chain, which initially contains ~70% of the total radioactivity of C3b, there is appearance of radioactivity associated with polypeptide chains of 68 000, 46 000 and 43 000 app. M_r. Whereas the radioactivity associated with the 68 000 and 43 000 M_r bands increases during the incubation period, the radioactivity of the 46 000

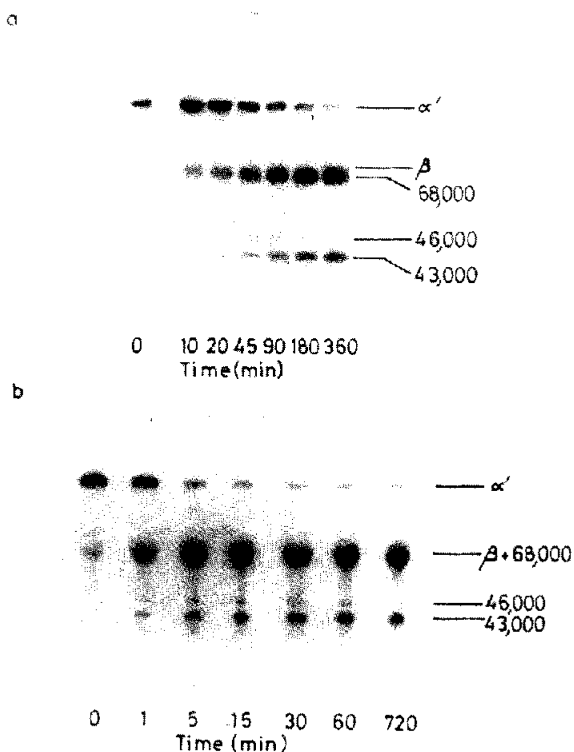

Fig.1. Pattern of degradation of ^{125}I-labelled C3b. Radio-iodinated C3b was incubated with either isolated C3b/C4b INA + β_1H or with human serum as in section 2. Samples were removed at various times as indicated. Autoradiographs of SDS–PAGE of reduced and alkylated samples are shown in both frames: (a) ^{125}I-labelled C3b (45 μg), β_1H (36 μg) and C3b/C4b INA (0.15 μg); (b) ^{125}I-labelled C3b (12 μg) in 10% (v/v) human serum.

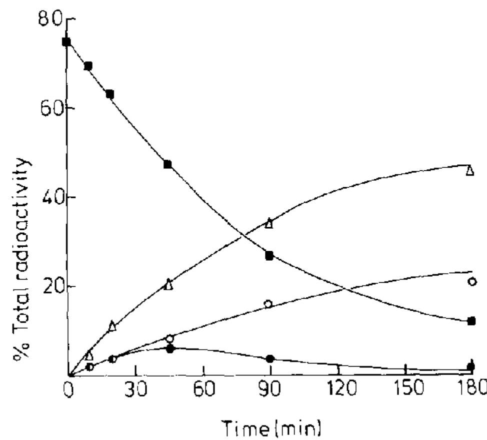

Fig.2. Time-course of production of C3bi. The gel tracks used to provide the autoradiograph shown in fig.1a were cut into 1 mm strips. The radioactivity associated with the α'-chain (■), 68 000 M_r fragment (△), 46 000 M_r fragment (●) and 43 000 M_r fragment (○) of the α'-chain have been expressed as a percentage of the total radioactivity in each track. Radioactivity associated with the 68 000 M_r band has been corrected for radioactivity due to the β-chain (25% of total at zero time).

M_r band increases initially and then decreases (fig.2). The pattern of appearance of radioactive bands (fig.2) shows that the 46 000 M_r species is the precursor of the 43 000 M_r product. In this system, therefore, the C3b α'-chain (108 000 app. M_r) is first cleaved to form the 68 000 and 46 000 M_r fragments. Further cleavage generates the 43 000 M_r fragment from the 46 000 M_r chain (for summary see fig.4). Neither SBTI nor DFP has any effect on the rate or pattern of cleavage of C3b.

Examination of cleaved C3b (C3bi) by SDS–PAGE without prior reduction shows that the β-chain, 68 000 and 43 000 (or 46 000) M_r fragments remain disulphide-linked to each other.

3.2. *C3b degradation in serum*

When ^{125}I-labelled C3b is incubated with human serum, the α'-chain is degraded rapidly (fig.1b) and the degradation pattern is identical to that seen in the purified system (fig.1a). The band at 68 000 M_r in fig.1b is distorted, as is the β-chain of C3b (70 000 M_r) because of the overload of albumin from serum which migrates in the same position. As in the purified system, SBTI and DFP have no effect on these stages of C3b breakdown in human serum (table 1).

Table 1
Rate of C3bi production in serum

Serum	Additions	Half-life of C3b α'-chain
Normal serum	–	0.6– 2.0 min
Normal serum	DFP	0.6– 2.0 min
Normal serum	SBTI	0.6– 2.0 min
Serum depleted of β_1H	–	22.5 h
Serum depleted of β_1H	β_1H	4.0– 9.0 min
Serum depleted of C4bp	–	4.0–15.0 min
Serum depleted of C4bp	C4bp	4.5–10.0 min
Serum depleted of β_1H and C4bp	–	>24 h
Serum depleted of β_1H and C4bp	C4bp	>24 h
Serum depleted of β_1H and C4bp	β_1H	4.2 min
Serum depleted of β_1H and C4bp	β_1H + C4bp	6.5 min
Serum depleted of C3b/C4b INA	–	15 h
Serum depleted of C3b/C4b INA	C3b/C4b INA	4.6 min

The rate of cleavage of the α'-chain of ^{125}I-labelled C3b (final conc. 120 μg/ml) in 10% (v/v) human serum, incubated as in section 2, was determined from graphs drawn as shown in fig.2. The half-life is expressed as the time required for disappearance of 50% of the α'-chain. When only one value is shown results are the average of two determinations. Where a range of values is indicated, at least 4 independent experiments have been performed

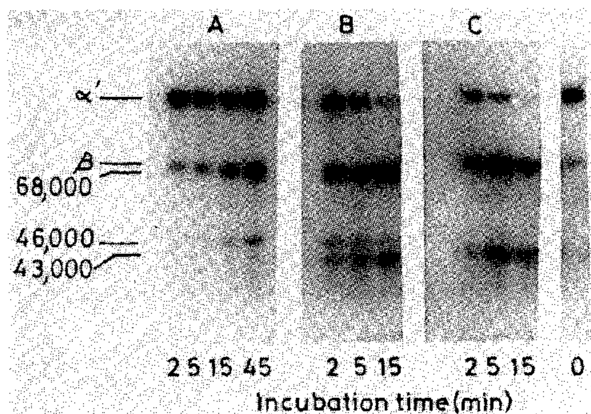

Fig.3. Effect of pH on cleavage of C3b. ^{125}I-Labelled C3b (0.5 μg, spec. act. 1.26 × 10^6 cpm/μg) was incubated for various times with β_1H (10 μg) and C3b/c4b INA (0.2 μg) in a composite buffer as in section 2: (a) pH 4.78; (b) pH 5.21; (c) pH 6.07. Autoradiographs of SDS–PAGE of reduced and alkylated samples are shown. The gel track on the extreme right shows C3b alone incubated at pH 4.68.

Even when ^{125}I-labelled C3b is incubated in 10% (v/v) human serum for up to 6 h at 37°C very little degradation of C3b beyond the C3bi stage is observed and from studies of prolonged incubation we have estimated that after 2 days at 37°C only 50% of C3bi is degraded further to C3c and C3d.

The degradation of ^{125}I-labelled human C3b to C3bi in sera from other species follows the same pattern as seen in fig.1. Both the 46 000 and 43 000 M_r fragments are observed when ^{125}I-labelled human C3b is incubated at 37°C in 5% (v/v) mouse, guinea pig and foetal calf serum. The double cleavage pattern appears in these other sera even although the rates of C3bi production differ. The half-life of human C3b in 5% (v/v) guinea pig, rabbit or foetal calf serum is <15 min, whereas in 5% (v/v) mouse serum it has a half-life of 2–3 h. In 5% (v/v) chicken serum, ^{125}I-labelled C3b was stable for at least 24 h.

When haemolytically active ^{125}I-labelled C3 is incubated with human serum the radio-labelled protein appears as intact C3 for at least 6 h. In contrast, ^{125}I-labelled C3, which has been treated with hydrazine to break the internal thiol ester bond in native C3, is degraded in human serum at the same rate as ^{125}I-labelled C3b. The α-chain of haemolytically inactive C3 is cleaved to 76 000 and 43 000 M_r fragments with the transient appearance of a 46 000 M_r fragment.

3.3. *Effect of pH on C3b cleavage*

The pH optimum for C3b/C4b INA activity is pH 6.0–6.5 [11,21] as measured by haemolytic assay. We have found a similar pH optimum for degradation of the α'-chain. At more acidic pH-values, the rate of the second cleavage appears to be selectively decreased. At pH 4.78 the 46 000 M_r species increases over the incubation period (fig.3a) with negligible production of the 43 000 M_r polypeptide. At pH 5.21, the 46 000 M_r species appears transiently and the amount of 43 000 M_r fragment increases throughout the incubation period (fig.3b). At the pH optimum for the overall reaction the 46 000 M_r species is very short-lived (fig.3c).

3.4. *Role of serum cofactors*

To test the physiological role of β_1H and C4bp in serum as cofactors in C3b breakdown to C3bi, the rate of degradation of the α'-chain of C3b was measured in human serum and human serum which had been depleted of β_1H and C3bp. In normal serum, C3b is degraded very rapidly to C3bi, with a half-life of ~1 min (table 1). The half-life of C3b in serum from which β_1H has been removed is increased dramatically to 22.5 h. Re-addition of β_1H at a physiological level to this serum decreases the half-life to

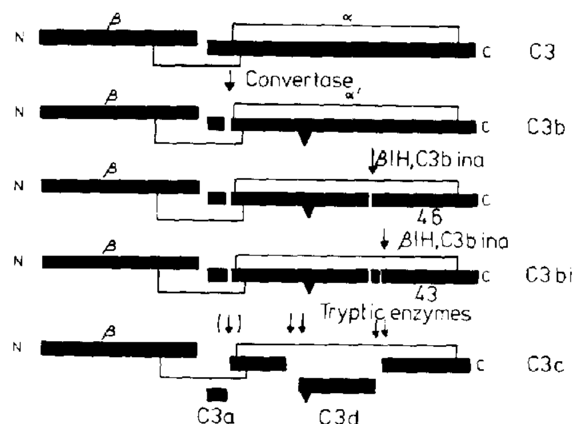

Fig.4. Summary of degradation of C3. Activation of C3 by C3 convertase cleaves the C3a fragment from the N-terminus of the α-chain and reveals the reactive acyl-group which is shown by the spike in the α'-chain of C3b. The spike also indicates the site of autolytic cleavage of C3 [27]. Two cleavages of C3b by β_1H and C3b/C4b INA are shown. The small fragment released from the 46 000 M_r polypeptide may come from either end of the 46 000 M_r fragment. Further degradation of C3bi by serum enzymes forms C3c, which consists of an intact β-chain and α'-chain fragments of ~25 000 and 38 000 M_r and C3d. The position and number of disulphide bridges, indicated by thin lines, is arbitrary.

~ 5 min. Removal of C4bp from serum increases the half-life of C3b slightly compared with normal serum but readdition of C4bp to this serum has no effect on the stability of C3b. C3b is extremely stable in serum which has been depleted of both β_1H and C4bp (half-life > 24 h) but is rendered much less stable (half-life ~ 5 min) when β_1H alone is restored. Re-addition of C4bp has no effect on the stability of C3b in serum depleted of both β_1H and C4bp, irrespective of whether β_1H has been restored. The residual cleavage of C3b in β_1H-depleted serum follows the same degradation pattern as shown in fig.1b and is likely to be due to <1% remaining β_1H.

It can be concluded from these results that C4bp does not have a significant role in C3b cleavage in human serum. In experiments with isolated proteins, however, C4bp does act as a cofactor in C3b cleavage, as in [6,7]. We have found that C4bp is ~5% as effective as β_1H on a weight basis. The double cleavage pattern of the α'-chain of C3b is again observed.

4. Discussion

These results show clearly in a purified system with C3b/C4b INA and β_1H that the α'-chain of C3b is cleaved at more than one peptide bond. The observed degradation pattern is the same whether pure C3b/C4b INA and β_1H are used or whether human or some other animal sera are used as the source of these proteins. The cleavage products are very likely to be the result of hydrolysis of only two peptide bonds but additional proteolytic removal of small oligopeptides from the main 68 000, 46 000 and 43 000 M_r fragments cannot be discounted. The double cleavage pattern occurs in a purified system and is not inhibited by DFP and SBTI. It is therefore unlikely that non-specific proteolysis by contaminating proteases (e.g., plasmin, kallikrein or thrombin) contributes to production of either the 46 000 or 43 000 M_r fragments.

The formation of C3bi described here is in agreement with the findings in [15]. The failure to observe the double cleavage of the α'-chain of C3b in [6,7, 10–14] may be explained by the transient appearance of the 46 000 M_r fragment, especially around neutral pH. We have found that the 46 000 M_r band is more readily observed by autoradiography than by Coomassie blue staining of SDS–polyacrylamide gels.

When C3b is generated from C3 an internal thiol ester in native C3 is hydrolysed. The thiolester bond can also be broken by treatment of C3 with nucleophiles and chaotropes without proteolysis, and haemolytically inactive C3 is produced. This inactive C3 has the same polypeptide chain structure as native C3 [20,22,23]. Inactive C3 can be further distinguished from native C3 in that it is susceptible to cleavage by C3b/C4b INA and β_1H ([11,24] and section 3). When inactive C3 is cleaved by C3b/C4b INA in the presence of β_1H, a 74 000 M_r fragment and a 46 000 M_r intermediate is produced which is further cleaved to a 43 000 M_r polypeptide. The 43 000 M_r fragment corresponds to the C-terminal portion of the α-chain of C3 [11,24,25]. The other, (74 000 M_r) fragment is eventually broken down in serum to form C3d [12].

The cleavage of inactive C3 by C3b/C4b INA and β_1H is completely distinct from the denaturation-induced autolytic cleavage of native C3 [17], although the denaturation-induced cleavage reaction does generate fragments of similar size (74 000 and 46 000 M_r) from the α-chain of C3. We have observed that on autolytic cleavage of ^{125}I-labelled active C3 that 66–70% of the radioactivity of the α-chain is associated with the small (46 000 M_r) fragment. In contrast, with C3b/C4b INA and β_1H-dependent cleavage of radio-iodinated inactive C3, only 25% of the radioactivity of the α-chain is associated with the 46 000 and 43 000 M_r fragments. These data support the conclusion that the autolytic cleavage point and the C3b/C4b INA cleavage sites, although producing similarly sized fragments, are at opposite ends of the α-chain of C3. The 46 000 M_r fragment produced by autolytic cleavage corresponds therefore to the N-terminal portion of the α-chain.

The C3d fragment derived by trypsin digestion of radio-iodinated C3 in agreement with [26] contains only 7–12% of the radioactivity of the C3 α-chain. By consideration of the content of radioactivity of the N-terminal and C-terminal fragments produced by the two types of cleavage discussed above, it is evident that C3d is derived from the central portion of the α-chain of C3. A summary of the degradation of C3 is shown on fig.4.

Acknowledgements

It is a pleasure to thank Mrs J. Hancox and Miss B. Moffat for their invaluable assistance. A. B. Wood received financial support from Humberside Local Education Authority.

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