Purification of human C3b inactivator by monoclonal-antibody affinity chromatography

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Monoclonal antibody has been obtained to the human complement control protein C3b inactivator after immunization of mice with the enzyme prepared by conventional methods. Antibody from ascitic fluid was purified and coupled to Sepharose-CL-4B to give a specific affinity column, which was used to isolate C3b inactivator from human serum in 70% yield. The product was characterized by size, chain structure, amino acid analysis and proteolytic activity.

The central event in the activation of the complement system by both pathways is the conversion of the third component, C3, into its activated form, C3b (reviewed by Reid & Porter, 1981). The C3 convertases catalysing this activation are complex, namely C4b2a in the classical pathway and C3bBb in the alternative pathway. Thus, in the latter case, the product C3b is also a component of the enzyme and in both cases the product can associate with the C3 convertase to give C5 convertase. In the classical pathway this is C4b2a3b, and in the alternative pathway, C3b_nBb. C3b can also bind to activating substances, such as aggregated antibody, antibody-coated cells or high-molecular-weight polysaccharides, and facilitate their uptake by phagocytic cells through C3b receptors.

The enzyme controlling the concentration of C3b, bound or free in blood, is therefore an essential part of the regulation of complement activation. Named C3b inactivator (C3bINA), it was first reported by Tamura & Nelson (1967) and Lachmann & Müller-Eberhard (1967, 1968). It has been shown to be a

proteinase which splits two bonds in the α' -chain of C3b (Harrison & Lachmann, 1980; Sim *et al.*, 1981) in the presence of a protein, cofactor H. C3bINA, with a different protein cofactor, C4b-binding protein, will also hydrolyse bonds in the α' -chain of C4b, where the specificity has been shown to be for Arg-Xaa peptide bonds (Press & Gagnon, 1981).

C3b inactivator has been isolated by conventional methods (Pangburn *et al.*, 1977; Fearon, 1977; Crossley & Porter, 1980), but though the last-cited method increased the yield substantially, it was still only 20% of the 35 mg/litre present in serum and required 2 weeks to complete. An improved method was sought, as the nature of the proteolytic activity, as judged by inhibition studies, was uncertain (Crossley & Porter, 1980). It is not inactivated by di-isopropyl fluorophosphate as are most serine proteinases. All other proteinases studied in the complement system are serine proteinases, though C2 and factor B are of a novel type (Christie *et al.*, 1980; Mole & Niemann, 1980; Christie & Gagnon, 1982). A more detailed investigation of the structure of C3bINA was necessary, and preparation by affinity chromatography using a monoclonal antibody to C3bINA has now been developed. This gives a yield of about 70% in 3 days.

Materials and methods

Materials

Outdated human plasma was obtained from the Churchill Hospital, Oxford, U.K.; Sepharose CL-4B and Sephacryl S-200 from Pharmacia (London); carrier-free ^{125}I and iodo[^3H]acetic acid from Amersham International, Amersham, Bucks., U.K.; iodo-gen from Pierce Chemical Co.; CNBr and pristane

Abbreviations used: the nomenclature used for the complement and control proteins is as recommended by the World Health Organisation (1968); C3bINA, C3b inactivator; C3b is the activation product of C3; cofactor H (previously known as $\beta_2\text{H}$), cofactor of C3b inactivator for the digestion of C3b; F(ab')₂ is the peptide digestion product of immunoglobulin G (IgG). Note: the Nomenclature Committee of the International Union of Immunological Societies (1981) recommends simply 'I' for C3bINA and 'H' for cofactor H ($\beta_2\text{H}$).

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(2,6,10,14-tetramethylpentadecane) from Aldrich Chemical Co., Poole, Dorset, U.K. DBA/2 $\times$ Balb/c F₁ mice were bred from the parent strains obtained from Olac 1976 Ltd., Bicester, Oxon., U.K. The NSI/1-Ag-4-1 non-secretor cell line was kindly provided by Dr. C. Milstein (Köhler & Milstein, 1976). ¹²⁵I-labelled rabbit F(ab')₂ anti-mouse IgG was prepared as described by Williams *et al.* (1977). Sheep anti-mouse immunoglobulin was purchased from Eivai Bios Laboratories Ltd., Horsham, Sussex, U.K.

Methods

Preparation of monoclonal antibodies. C3bINA was purified by the method of Crossley & Porter (1980). DBA/2 $\times$ Balb/c F₁ mice were injected twice (2 weeks apart) subcutaneously with 20 μ g of C3bINA per mouse in complete Freund's adjuvant. After 14 days the resultant sera were analysed by radioimmunoassay for antibodies against C3bINA. The mouse giving the serum with the highest titre (10000 c.p.m. at a dilution of 1:10000) was re-injected intravenously with 20 μ g of C3bINA. After 4 days the spleen was removed for fusion with the NS-1 myeloma line by the method of Galfre *et al.* (1977). After 9 days the tissue-culture supernatants of the hybrids were screened for antibodies reacting against C3bINA by radioimmunoassay and immunoprecipitation. One hybrid producing antibody against C3bINA was cloned by limiting dilution (Lernhardt *et al.*, 1978) and is called 'MRC OX 21'.

Radioimmunoassay to detect antibodies to C3bINA. A solid-phase indirect radioimmunoassay for C3bINA was constructed; 50 μ l of C3bINA (50 μ g/ml) in phosphate-buffered saline (Dulbecco's A; Oxoid Ltd., Basingstoke, Hants., U.K.) was added to each well of a 96-well vinyl microtitre plate (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.). After 30 min at room temperature the C3bINA solution was removed and could be used several times, since only a small percentage bound to the vinyl plate. Bovine serum albumin (1% in phosphate-buffered saline) was added to cover the whole plate and incubated for 10 min to block further non-specific binding of protein. Samples (25 μ l) containing antibody against C3bINA were added to each well and incubated for 30 min. The plate was washed with distilled water five times and ¹²⁵I-labelled rabbit F(ab')₂ anti-mouse IgG (50 μ l; 0.2 μ g/ml; 30 μ Ci/ μ g) was added to each well and incubated at room temperature for 30 min. After removal of the ¹²⁵I-labelled antibody and washing the plate five times with distilled water, the vinyl plate was cut up and the ¹²⁵I in each well determined with an LKB Rackgamma counter (LKB Instruments Ltd., Selsdon, Surrey, U.K.).

Radioimmunoassay to quantify C3bINA. The

amount of C3bINA in fractions during the purification was determined by inhibition of the above radioimmunoassay as follows. Samples of dilutions of C3bINA (100 μ l) were incubated with 100 μ l of the monoclonal anti-C3bINA antibody (called 'MRC OX 21 antibody') diluted 1:20 in phosphate-buffered saline. After 1 h at 4°C the absorbed antibody (50 μ l in duplicate) was added to each well of a vinyl microtitre plate which had been precoated with C3bINA as described above. After 1 h at 4°C the plate was washed and incubated with ¹²⁵I-labelled rabbit F(ab')₂ anti-mouse IgG as described above, except that incubation was for 1 h at 4°C.

Immunoprecipitation of C3bINA. C3bINA purified as in Crossley & Porter (1980) was labelled with ¹²⁵I by using 25 μ g of iodogen, 60 μ g of C3bINA and 1 mCi of ¹²⁵I by the method of Fraker & Speck (1978). The specific radioactivity of the radioiodinated C3bINA was 6.7×10^6 c.p.m./ μ g. To minimize background radioactivity in immunoprecipitation, 200 μ l of ¹²⁵I-labelled C3bINA (20 μ g/ml in 5 mM-veronal/0.15 M-NaCl/1 mM-MgCl₂/1 mM-CaCl₂ buffer, pH 7.5) was incubated for 2 h at 0°C with 230 μ g of mouse IgG which was completely precipitated with sheep anti-mouse IgG. After centrifugation the supernatant was used for specific immunoprecipitation: ¹²⁵I-labelled C3bINA (6.7×10^5 c.p.m.) was incubated with 60 μ l of tissue-culture supernatant at 0°C for 4 h. Mouse IgG (5.2 μ g) and an amount of sheep anti-(mouse IgG) serum optimum for precipitation were added and incubated at 4°C overnight. The precipitates were washed three times in 0.15 M-NaCl and twice in 0.5% sodium deoxycholate/0.01 M-Tris/HCl, pH 8.0, before analysis by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, and autoradiography (Laemmli, 1970).

Purification of C3bINA by affinity chromatography. DBA/2 $\times$ Balb/c F₁ mice were treated with pristane and 3 weeks later given 10⁷ MRC OX 21 hybrid cells intraperitoneally per mouse. After 2 weeks, ascitic fluid was collected and immunoglobulin was prepared by precipitation with 20% Na₂SO₄ and ion-exchange chromatography on DEAE-cellulose DE-52 (Whatman Ltd., Maidstone, Kent, U.K.) in 0.05 M-NaCl/0.025 M-Tris/HCl, pH 7.4. The IgG with anti-C3bINA activity was coupled to Sepharose CL-4B at the level of 10 mg of protein/ml of beads by using CNBr activation as described by Porath (1974).

The affinity-chromatography steps were at 4°C and all buffers contained 5 mM-NaN₃. Plasma (700 ml) to which 0.7 ml of 2.5 M-di-isopropyl fluorophosphate in propan-2-ol was added, was centrifuged at 10000 g for 30 min. The supernatant was applied first to a 10 ml Sepharose CL-4B column coupled with 10 mg of bovine IgG/ml of beads and then in sequence to a column containing 190 mg of

MRC OX 21 antibody at a flow rate of approx. 30 ml/h. The MRC OX 21-antibody column was washed with 0.14 M-NaCl/0.025 M-Tris/HCl/0.02 M-EDTA, pH 7.3, and the NaCl concentration was increased to 0.5 M for approx. 100 ml until the A_{280} was less than 0.03. The bound antigen was eluted with 0.14 M-NaCl/0.05 M-diethylamine/HCl, pH 11.5, and the fractions were neutralized with solid glycine to pH 8.2 (Sunderland *et al.*, 1979). After dialysis against 0.15 M-NaCl/0.025 M-Tris/HCl, pH 7.3, the eluted protein was concentrated by ultrafiltration with an Amicon PM10 membrane or by precipitation with 60%-satd. $(\text{NH}_4)_2\text{SO}_4$ and was chromatographed on a column (3 cm $\times$ 100 cm) of Sephacryl S-200 in the same buffer.

Separation of C3bINA polypeptide chains. C3bINA (200 nmol) was dialysed against 0.1 M-Tris/HCl buffer, pH 8.0, and dithiothreitol (30 nmol) added to give a final concentration of 1.5 mM. After incubation at 37°C for 1 h, iodo[^3H]acetic acid (312 μCi ; 61 mCi/mmol) was added. After 5 min at room temperature, unlabelled iodoacetic acid was added to give a final concentration of 3.3 mM and the reaction mixture was incubated at 37°C for 30 min. The reduced and alkylated sample was dialysed against distilled water and then against 0.1 M-acetic acid, before freeze-drying. It was dissolved in 0.2 M-formic acid/6 M-urea and fractionated by high-pressure liquid chromatography, as described by Arlaud *et al.* (1982), in the same solvent by using a gel-filtration column (TSK-G 3000 SW; 7 mm $\times$ 600 mm) purchased from LKB. The flow rate was 0.6 ml/min and protein elution was measured in an LKB Uvicord S monitor at 276 nm.

Measurement of C3bINA proteolytic activity. To measure the splitting of the α' -chain of C3b catalysed by C3bINA, human plasma (5 μl) or C3bINA-depleted plasma (5 μl) was mixed with ^{125}I -labelled C3b (0.5 μg ; specific radioactivity 1.2×10^6 c.p.m./ μg) in a total volume of 100 μl of 20 mM-sodium phosphate/140 mM-NaCl, pH 7.4. Samples of purified fractions of C3bINA (0.2–0.4 μg) were added to C3bINA-depleted plasma in the same buffer. After incubation for various times at 37°C, 20 μl samples were removed and were fractionated by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate-containing buffers. The percentage cleavage of the C3b α' -chain was measured (Sim *et al.*, 1981).

To compare the proteolytic activity of different preparations of C3bINA, the rate of hydrolysis of the α' -chain was measured in the presence of excess pure cofactor H (Sim *et al.*, 1981).

Other methods. The protein concentration of purified C3bINA was calculated by using $A_{280}^{1\text{cm}} = 1.0$ for a 1 mg/ml solution (Crossley & Porter, 1980). Amino acid analyses were performed with an LKB 4400 amino acid analyser after 24, 48 and 72 h

hydrolysis as described previously (Campbell *et al.*, 1981).

Results

Preparation of monoclonal antibodies against C3bINA

C3bINA was purified by the method of Crossley & Porter (1980) and used to immunize mice for the production of monoclonal antibodies. An indirect radioimmunoassay was developed to quantify mouse antibodies specific for C3bINA by passively adsorbing purified C3bINA on vinyl microtitre plates, allowing it to react with the mouse antibodies, which were in turn detected with ^{125}I -labelled rabbit F(ab') $_2$ anti-(mouse IgG) antibodies. The assay was used during cloning of the monoclonal antibodies resulting from one fusion. These antibodies were also screened by immunoprecipitation of ^{125}I -labelled C3bINA. One monoclonal antibody was obtained which precipitated C3bINA. The precipitate gave the two characteristic polypeptide chains of C3bINA after reduction, when it was analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and autoradiography. This antibody was used in all the studies reported here and is called 'MRC OX 21 antibody'.

The MRC OX 21 antibody was also used to assay C3bINA during purification studies by comparing the ability of fractions to inhibit the binding of this antibody to C3bINA. This is illustrated in Fig. 1, which shows inhibition of the assay by purified C3bINA and human plasma. Comparison of the amount of protein required for 50% inhibition of this assay gives an estimate of 37 μg of C3bINA/ml of serum, a result which is comparable with that reported by others (Pangburn *et al.*, 1977).

Fig. 1. Inhibition radioimmunoassay for C3bINA. The assay was carried out as described under 'Methods' and shows the inhibition assay for human plasma (●) and for C3bINA (○) purified as described by Crossley & Porter (1980).

Table 1. *Purification of C3bINA by affinity chromatography from 700 ml of plasma*

The values given are an average for three preparations. (a) Starting antigenic activity was 4.5×10^5 units, where one unit is defined as the amount necessary to give 50% inhibition of the assay described under 'Methods'. (b) Proteolytic activity is measured as percentage of C3b α' -chain hydrolysed/min in the presence of excess cofactor H.

Fraction	Total protein (mg)	(a)	(b)	Purification factor	
		Total C3bINA content by radioimmunoassay (%)	Total proteolytic activity (%)	Antigenic	Proteolytic
Plasma	43 500	100	100	1.0	1.0
Eluted from affinity column	39.2	77	83	850	920
From gel filtration	16.7	64	73	1670	1900

Fig. 2. Fractionation of C3bINA by affinity chromatography on a column of Sepharose CL-4B to which had been coupled the monoclonal antibody MRC OX 21 as described under 'Methods'

O, A_{280} ; ●, radioimmune assay of C3bINA.

Purification of C3bINA

In a typical preparation of C3bINA, 700 ml of human plasma was applied to an affinity column containing 190 mg of monoclonal MRC OX 21 antibody coupled to Sepharose CL-4B and the bound protein eluted by pH 11.5 buffer. Protein was determined by A_{280} and C3bINA by inhibition of the radioimmunoassay to give the profile observed in Fig. 2 and the recoveries and purification in Table 1. All the C3bINA protein antigenic activity was removed by the affinity column and it was eluted in good yield. The C3bINA enzymic activity was also removed by this column (result not shown) and was recovered in the eluted fraction (see below). The first fraction containing protein eluted from the affinity

Fig. 3. Polyacrylamide-gel electrophoresis, in sodium dodecyl sulphate-containing buffers, of purified C3bINA preparation

(a) and (b), C3bINA purified by affinity chromatography and gel filtration. (c) and (d), C3bINA purified by affinity chromatography alone. Samples (a) and (c) were unreduced; samples (b) and (d) were reduced and alkylated. The apparent-molecular-weight calibration was based on standards of the reduced proteins: myosin (200 000), β -galactosidase (116 000), phosphorylase *a* (94 000), bovine serum albumin (68 000), IgG heavy chain (50 000), ovalbumin (43 000) and IgG light chain (25 000).

column contained little antigenic activity (Fig. 2). This probably represents denatured and aggregated C3bINA, as the pooled material was almost pure when analysed by polyacrylamide-gel electrophoresis (Fig. 3), giving major bands with identical

Table 2. Amino acid composition of the peptide chains of C3b inactivator

Amino acid	Amino acid composition (residues/100 residues)	
	Heavy chain	Light chain
Cys	5.6	4.1
Asp	9.7	11.5
Thr	6.8	4.5
Ser	7.1	5.7
Glu	11.9	9.8
Pro	4.6	4.7
Gly	7.5	10.1
Ala	5.5	5.8
Val	7.7	7.7
Met	1.5	1.1
Ile	3.2	7.1
Leu	7.2	5.4
Tyr	3.2	5.3
Phe	3.9	3.6
His	2.3	2.3
Lys	8.0	7.0
Arg	4.0	4.1

mobilities compared with C3bINA purified by the method of Crossley & Porter (1980). Fig. 3 shows that the apparent molecular weight of the unreduced C3bINA was about 75 000 compared with the value of 90 000 previously given when non-reduced markers were used (Crossley & Porter, 1980). The minor contaminants and aggregated C3bINA were removed by a final gel-filtration step to raise the specific activity considerably, to give a final yield of about 70% (Fig. 3 and Table 1).

Characterization of C3bINA

The C3bINA purified by affinity chromatography retained enzymic activity, as shown by its ability to cleave C3b and C4b in the presence of the appropriate cofactors, and its specific activity was somewhat higher than that prepared by the method of Crossley & Porter (1980).

The two polypeptide chains of C3bINA were separated by high-pressure liquid chromatography after reduction and alkylation. The amino acid analysis of each chain is given in Table 2. As both chains contain carbohydrate, the number of amino acid residues per chain is uncertain.

Discussion

The use of a monoclonal antibody MRC OX 21, which recognizes the C3bINA protein in an affinity column, gave a very rapid purification of C3bINA from human plasma at high yield. Typically, 15–20 mg of C3bINA could be rapidly purified from

700 ml of plasma in 3 days compared with 6 mg from 500 ml of rat plasma in 2 weeks by conventional techniques (Crossley & Porter, 1980). The affinity column used was saturated by about 800 ml of plasma, giving an efficiency of more than 10% of the theoretical value, assuming that all the antibody on the column was active and able to bind through both antibody-combining sites. The column has been re-used several times with no detectable loss in efficiency. The C3bINA eluted by the high-pH step was enzymically active with a specific activity a little higher than reported previously (Crossley & Porter, 1980). This purification procedure can readily provide the amounts of purified protein necessary for amino acid sequence studies. These have now shown that C3bINA is a serine proteinase, though probably with unusual features (Hsiung *et al.*, 1981). It is apparent from the amino acid analysis that the smaller catalytic chain contains an exceptionally high content of cystine compared with the catalytic chains of other serine proteinases, suggesting the presence of at least one additional disulphide bond.

The use of a monoclonal antibody to purify enzymically active C3bINA demonstrates that this technique could be of value in the purification of other serum proteins in low concentration such as the complement components C2 and Factor D. The small number of purification steps in this method is advantageous with respect to time, yield and retention of enzymic activity.

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