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Bovine conglutinin binds to an oligosaccharide determinant presented by iC3b, but not by C3, C3b or C3c

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SUMMARY

Bovine conglutinin is a serum lectin that agglutinates erythrocytes preincubated with antibodies and complement. This agglutination occurs through the binding of conglutinin to iC3b, a fragment of the complement component C3. It was reported that conglutinin binds fluid-phase C3b and C3c as well as iC3b. We re-investigated the reactivity of conglutinin towards fluid-phase C3 degradation products. ELISA wells were coated with conglutinin and reacted with C3 split products generated in normal human serum, in factor I-deficient serum, or in factor I-depleted serum. Conglutinin-bound C3 fragments were detected with anti-C3c and anti-C3d antibodies. An increased signal was observed during the activation of complement in normal human serum with the peak response after 1–2 hr, following which the signal decreased, reaching background level after 72 hr. The oligosaccharides on C3c, generated in serum, are thus not recognized by conglutinin. No signal was observed when factor I-deficient serum or factor I-depleted serum was used instead of normal serum. Reconstitution with purified factor I re-established the normal pattern. Examination of the conglutinin-bound C3 molecules by SDS–PAGE and Western blotting with anti-C3c and anti-C3d antibodies revealed bands characteristic for iC3b, and no bands corresponding to C3b or C3c. Reduction of the disulphide bonds prior to the incubation of the activated serum with the conglutinin-coated wells revealed a band of 63,000 MW, characteristic of the N-terminal fragment of the α-chain of iC3b. We also investigated the binding to the solid-phase conglutinin of purified C3 and degradation products generated with enzymes. In this case, C3 as well as C3b and C3c were bound, suggesting conformational changes in C3 during purification. In conclusion, when C3 conversion takes place at near physiological conditions, conglutinin interacts specifically with the oligosaccharide on the α-chain of iC3b.

INTRODUCTION

Conglutinin was described many years ago as a bovine serum protein that was able to agglutinate erythrocytes that had been reacted with antibodies and complement.[1] Lachmann & Müller-Eberhard showed that the interaction was dependent on a factor in the serum, which they termed conglutinogen-activating factor (KAF).[2,3] This factor is now known as factor I and it has been shown to be a serine protease which, in the presence of co-factors, cleaves the α-chain of C3b at two closely spaced sites, generating the inactivated form of C3 termed iC3b (Fig. 1). Prolonged incubation of antibody-coated erythrocytes with serum prior to the incubation with conglutinin abolishes the agglutination.[2] During prolonged incubation with serum, the iC3b is cleaved to C3d and C3c. This specific interaction with erythrocyte-associated iC3b has been confirmed by others.[4–6] The binding between conglutinin and iC3b is calcium-dependent and can be inhibited by monosaccharides, with *N*-acetyl-D-glucosamine (GlcNAc) being most potent.[7,8] Thus, conglutinin is the first animal lectin described. Conglutinin shows selective reactivity with the high mannose *N*-linked oligosaccharide on the α-chain of iC3b.[9] Conglutinin also reacts with yeast carbohydrates presented on yeast cell-wall preparations (zymosan) and soluble yeast glycoproteins (mannan) as well as other oligosaccharide structures.[7,10,11]

The biological role of conglutinin is unresolved, but several reports indicate a function in the immune system. Decreased levels of conglutinin in serum are seen in connection with abortion, calving and infection,[12,13] and an anti-bacterial effect towards Gram-negative bacteria,[14–17] as well as an antiviral effect against influenza A virus and human immunodeficiency (HIV)[18–22] have been described.

In 1985 it was reported that conglutinin binds not only iC3b, but just as avidly C3b and C3c.[23] In contrast to the earlier investigations with erythrocyte-associated C3, this work was done using fragments of C3 present in the fluid phase. The C3-

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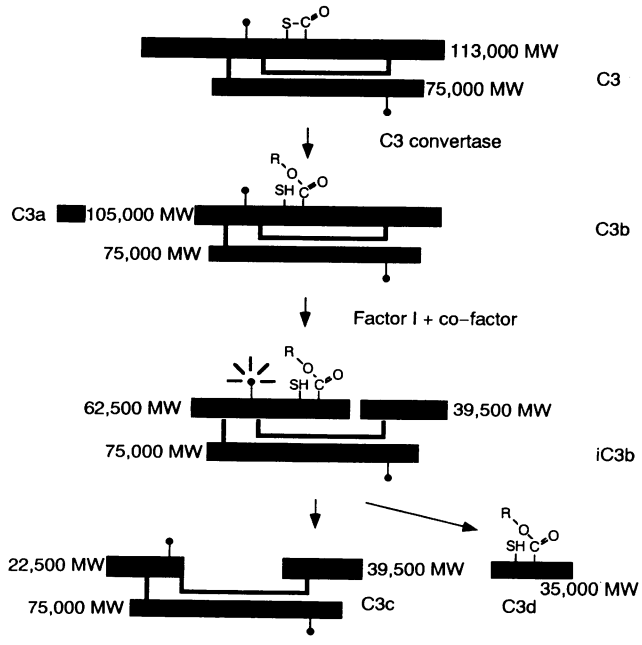

Figure 1. Diagram showing the activation and degradation of complement component C3. The two high mannose-type oligosaccharides are indicated by (ᚠ) with the conglutinin reactive one highlighted.

split products were generated by treatment of purified C3 with enzymes rather than in a serum milieu. The results appear to indicate a different specificity of conglutinin towards the membrane-bound C3-split products and the fluid-phase C3-split products. The aim of the present work was to re-investigate this discrepancy.

MATERIALS AND METHODS

Conglutinin

Bovine blood was collected at a local abattoir and conglutinin was precipitated from bovine serum by the addition of 3·5% polyethylene glycol (PEG) 6000 (w/v) (Sigma, St Louis, MO). The precipitate was redissolved in 200 ml column buffer (5 mM barbital, 145 mM NaCl, 15 mM NaN_3, 2 mM $CaCl_2$, pH 7·4) and applied to a column (70 ml) of *N*-acetyl-D-glucosamine (GlcNAc, code A 8625; Sigma) coupled to Sephacryl S-300 beads (Pharmacia, Uppsala, Sweden) according to Fornstedt & Porath.[24] The column was washed with 500 ml column buffer and conglutinin was eluted with the same buffer containing 10 mM EDTA instead of $CaCl_2$.

The eluate was recalcified, the pH adjusted to pH 7·4 and applied to the same column again. After washing the column, conglutinin was eluted with column buffer containing 2 mM GlcNAc. The eluate (20 ml) was dialysed against TBS–EDTA (10 mM Tris, 140 mM NaCl, 10 mM EDTA, 15 mM NaN_3, pH 7·4) to remove the bound GlcNAc, and then against TBS (10 mM Tris, 140 mM NaCl, 15 mM NaN_3, pH 7·4). To remove anti-carbohydrate antibodies, the eluate was passed through a column consisting of CNBr-activated Sepharose 4B (Pharmacia) coupled with rabbit anti-bovine Ig antibody (code Z 247; Dakopatts, Glostrup, Denmark). The purified material was analysed by SDS–PAGE and silver staining, and found to be more than 95% pure.

Factor I-depleted serum, factor I-deficient serum and factor I

Ten millilitres of normal human serum (NHS) was passed at 4° through a column containing rabbit anti-human factor I antibodies[25] coupled to Sepharose 4B. The affinity matrix was made by the coupling of dissociated immune complexes (generated by mixing normal human serum with rabbit anti-human factor I antiserum).[25] The effluent was collected (factor I-depleted serum) and the column was washed with TBS + 0·05% (v/v) Tween 20 (TBS–Tween). Factor I was eluted with 0·1 M glycine–HCl, pH 2·5, and immediately neutralized with 1 M Tris, pH 8·0. The effluent and the eluate were analysed for factor I by rocket immunoelectrophoresis.[26] Total depletion of factor I in the effluent was observed. No conversion of C3 to C3c in the effluent could be observed upon analyses by crossed immunoelectrophoresis.[26] Factor I-deficient serum was obtained from two people lacking functional factor I.[26]

Activation of the complement system

The complement system was activated in samples of sera from two healthy persons, two factor I-deficient patients and the factor I-depleted serum by the addition of 1 vol of Sephadex G25 superfine beads (Pharmacia) suspended at 50% (wet wt/v) in B1 buffer (5 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7·4), to 1 vol of serum. The G25 beads had been hydrated and washed with the B1 buffer prior to the incubation with serum. The mixtures were placed at 37° on a rotor and the activation was stopped after different incubation times by the addition of 20 vol ice-cold TBS, and the G25 beads pelleted by centrifugation (200 ***g*** for 5 min at 4°). The supernatants were either analysed immediately or frozen at −70°. The effect of factor I on complement activation was also demonstrated by the addition of 10 mM $ZnCl_2$, which inhibits the function of factor I.[27]

To examine if the C3 in the serum incubated for 1 week at 37° with Sephadex G25 had been fully degraded to C3c, crossed immunoelectrophoresis was performed against rabbit anti-C3c antibody (diluted 1/800) or rabbit anti-C3d antibody (diluted 1/300) (Dakopatts A062 and A063), essentially as described elsewhere.[26]

Reduction and alkylation of serum samples

After stopping complement activation with ice-cold TBS, the supernatants were reduced by the incubation at 4° for 1 hr with 40 mM dithiothreitol followed by alkylation by the addition of 90 mM iodoacetamide.

Purification of C3 and generation of C3b, C3c and C3d

C3 was purified from normal human serum as described elsewhere.[28] Briefly, 50 ml human plasma (fresh-frozen plasma, obtained from the Regional Blood Transfusion Service, John Radcliffe Hospital, Oxford, U.K.) containing 10 mM EDTA and 0·5 mM Pefabloc-SC (Pentopharm AG, Basel, Switzerland) was precipitated with 5% PEG 3350 (Sigma no. P3640) and the proteins in the supernatant were fractionated on Q-Sepharose (Pharmacia). Fractions containing C3, identified by SDS–PAGE analysis, were then further purified on a FPLC Mono-Q column (Pharmacia). The C3 obtained was >95% pure, as assessed by SDS–PAGE analysis.

C3 was digested with 1% w/w trypsin (Sigma no. T2271) for 90 seconds at 37° in order to generate C3b.[23] Trypsin activity was inhibited by adding 2% w/w soybean trypsin inhibitor

(Sigma no. T9003). C3c and C3d were generated from purified C3 either by addition of 2% w/w trypsin, incubation at 37° for 2 hr followed by addition of excess of soybean trypsin inhibitor (4% w/w), or by addition of 5% elastase (Sigma no. E0127) followed by incubation at 37° for 5 hr.[23]

ELISA

Microtitre wells (Maxisorb Immunoplates, Nunc, Kamstrup, Denmark) were coated overnight at room temperature with 0·5 μg purified conglutinin in 100 μl coating buffer (0·1 M sodium carbonate, pH 9·6). The wells were blocked for 1 hr with bovine serum albumin (BSA; Sigma no. A 7517) at 1 mg/ml TBS, and were then washed three times with TBS–Tween. If the plates were not used immediately the wells were emptied and filled with TBS. Between all further steps, the wells were washed with B2 buffer [5 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 0·05% (v/v) Tween 20]. All dilutions and washes were done with ice-cold buffers and on melting ice to prevent further complement activation. Incubations were at 4°. The serum samples were applied at a final serum dilution of 1/1000 in B2 buffer or, as a control, in B3 buffer (5 mM barbital, 145 mM NaCl, 10 mM EDTA, 0·05% Tween 20). The factor I-deficient sera were diluted only 1/100 because these sera contained only about 10% the normal C3 level, as determined by rocket immunoelectrophoresis (data not shown). Purified C3 and degradation products thereof, generated with trypsin or elastase, were incubated at concentrations of 1, 0·33 and 0·11 μg/ml B2 buffer or B3 buffer (control).

The microtitre plate with samples was incubated for 2 hr at 4° and, after washing with B2 buffer, the wells received 0·3 μg rabbit anti-C3c or 0·3 μg rabbit anti-C3d antibodies in 100 μl B2 buffer. After 2 hr of incubation, the wells were washed and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma no. A8025) diluted 1/5000 in B2 buffer was added. The incubation was continued for 2 hr, then the wells emptied and washed. Development was with 0·1 mg p-nitrophenyl phosphate, (code no. 107905; Boehringer Mannheim, Germany) in DEA buffer (0·1 M diethyl amine, pH 9·8). The plates were incubated at 37° and the absorption read at 405 nm using a Biorad model 450 immunoreader.

SDS–PAGE and Western blotting

C3 fragments bound to the solid-phase conglutinin were eluted and analysed by electrophoresis. The elution was performed sequentially from a total of 16 wells per electrophoresis sample using the following procedure. Two wells were emptied, washed and 100 μl B3 buffer added per well. After 5 min the next two wells were emptied, washed and the elution buffer was transferred to these wells from the first two wells. This was repeated eight times in total and the 200-μl sample was reduced and denatured by heating at 100° in 40 mM dithiothreitol (DDT), 1·5% (w/v) SDS, 5% (v/v) glycerol, 0·1 M Tris, pH 8·0, and alkylated by the addition of 90 mM iodoacetamide. The samples were run on 150 × 150 × 0·75 mm, 4–20% polyacrylamide gradient gels using the Laemmli discontinuous buffer system.[29] The molecular weight standards were Rainbow™ coloured protein molecular weight markers (no. RPN 755, Amersham, U.K.). The gel with samples coming from conglutinin-coated wells reacted with reduced and alkylated serum samples was silver stained.[30] The gel with proteins from wells incubated with non-reduced serum samples was blotted onto a polyvinylidene-difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA). The Western blot was blocked in TBS containing 0·1% Tween 20 for 15 min followed by incubation with rabbit anti-C3c at 5 μg/ml TBS–Tween. The blot was washed three times in TBS–Tween and incubated with alkaline phosphatase-conjugated goat anti-rabbit Ig (Sigma no. A8025) diluted 1/5000 in TBS–Tween. After additional washing, the blot was developed with 100 μg nitroblue tetrazolium (Sigma no. N6876) and 50 μg potassium-5-bromo-4-chloro-3-indolylphosphate (Sigma no. B6274)/ml 2 mM $MgCl_2$, 0·1 M ethanolamine, pH 9·0. After photographing the blot, it was developed with anti-C3d antibody at 4 μg/ml, as described above.

RESULTS

The complement system in NHS, factor I-deficient serum and factor I-depleted serum was activated by incubation with Sephadex G25, and the binding of C3 or C3 fragments to solid-phase conglutinin was evaluated with rabbit anti-C3c or anti-C3d antibodies. The results in Fig. 2 show that the amount of C3 fragments from NHS bound to conglutinin reached a maximum after 1–2 hr of incubation with Sephadex G25 and showed a slow decline upon prolonged incubation. No conglutinin-reactive C3 fragments were generated in the NHS if the incubation of serum with Sephadex G25 was performed in the presence of EDTA, which inhibits both the classical and the alternative complement cascade, or when the incubation was performed in the presence of zinc ions, which inhibit the activity of the factor I co-factor, factor H (Fig. 2). Furthermore, no conglutinin-reactive C3 fragments were generated in factor I-deficient serum, in fully complement converted serum or in NHS depleted of factor I (Fig. 2). If the factor I-depleted serum was reconstituted by the addition of purified factor I, a response similar to that obtained for NHS was observed. The kinetics were the same with the two detecting antibodies, anti-C3c and anti-C3d.

To investigate if the C3 molecules in the fully converted serum had been degraded to C3c, crossed immunoelectrophoresis was performed against anti-C3c antibody. Only C3c could be detected in this serum (data not shown).

If purified C3 and degradation products thereof generated with elastase or trypsin were added to the conglutinin-coated wells instead of the activated serum, binding of C3, C3b and C3c could be detected. In this assay, conglutinin interacted most strongly with intact C3 (data not shown).

To further analyse the nature of the C3 degradation product bound to conglutinin, a Western blotting analysis was employed. The bound C3 fragments were eluted from the conglutinin-coated wells by 10 mM EDTA. The eluate was subjected to SDS–PAGE followed by Western blotting and the blot was developed with anti-C3c antibody. The results in Fig. 3a show the appearance of two bands of 75,000 and 40,000 MW after the complement system had been activated for 10–15 min. Maximal intensity of the bands was reached after an activation time of 45 min, and a decrease observed upon prolonged activation. The kinetics are similar to those observed in the ELISA experiment. The different intensities of the two bands are likely to reflect differences in the amounts of antibodies reactive against the two polypeptide chains. The same Western blot was then reacted with anti-C3d antibody, and an additional band with a MW of about 63,000 was observed with the same

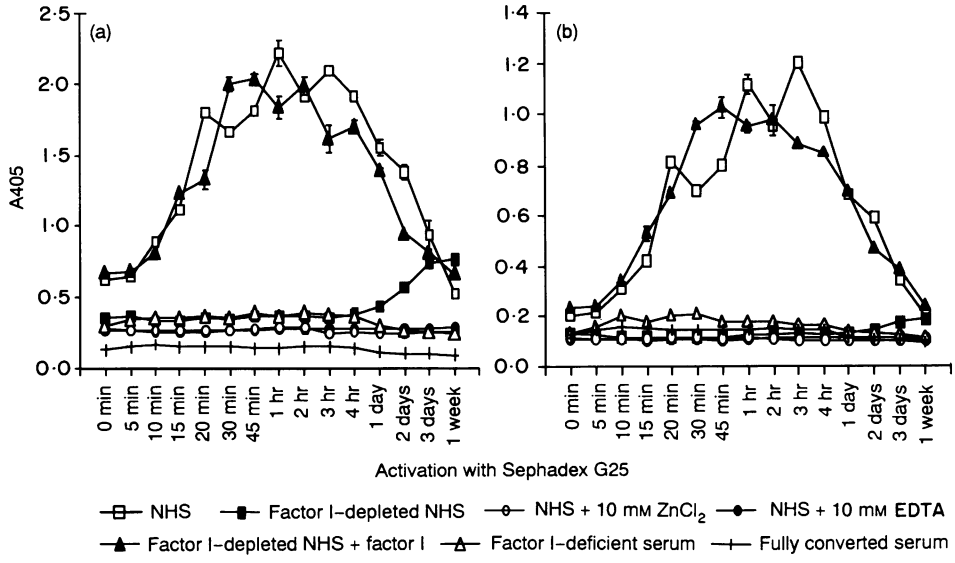

Figure 2. Generation of conglutinin-reactive C3 fragments in serum. After incubation of the sera with Sephadex G25 for various time periods, samples were transferred to conglutinin-coated wells and bound C3 fragments were detected with anti-C3c antibody (a) or anti-C3d antibody (b).The sera were: NHS; factor I-depleted serum, NHS depleted for factor I by affinity-chromatography; NHS with 10 mM $ZnCl_2$ added to inhibit factor I; NHS + 10 mM EDTA; factor I-depleted NHS + factor I (factor I-depleted NHS reconstituted with purified factor I); factor I-deficient serum (serum from a patient deficient in factor I); fully converted serum (serum incubated for 1 week at 37°).

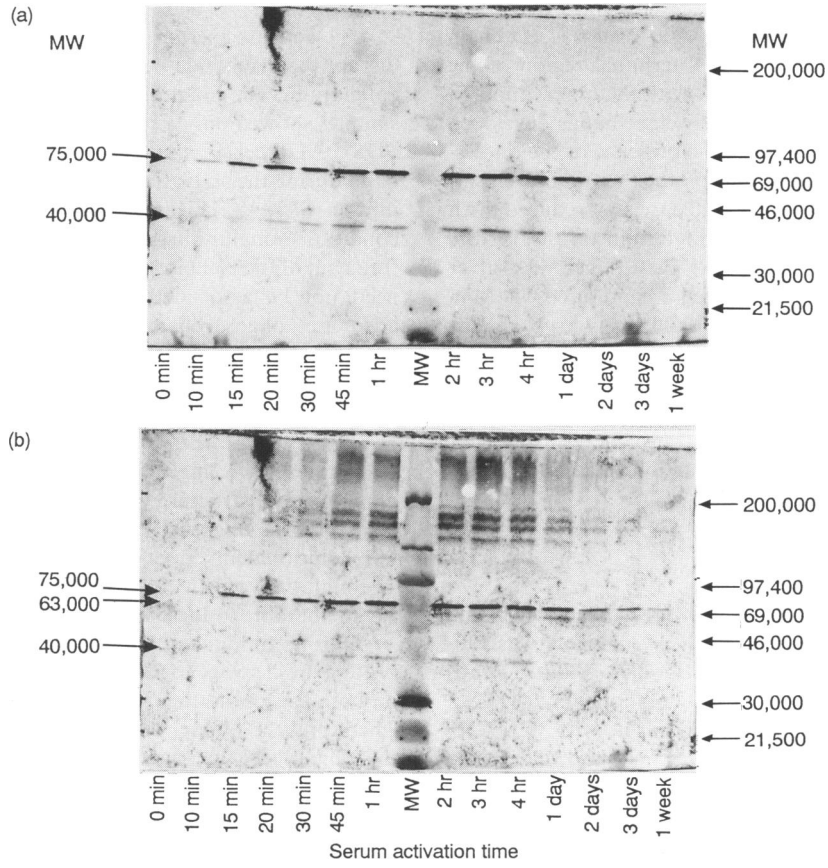

Figure 3. Western blot analysis of eluates from conglutinin-coated wells incubated with Sephadex G25-activated NHS. The blot was probed with anti-C3c (a) and then the same blot was probed with anti-C3d (b). Eluates from wells incubated with serum activated for increasing time periods were run in the different lanes. The molecular weight markers (MW) were: myosin (200,000 MW), phosphorylase b (97,400 MW), bovine serum albumin (69,000 MW), ovalbumin (46,000 MW), carbonic anhydrase (30,000 MW) and trypsin inhibitor (21,500 MW).

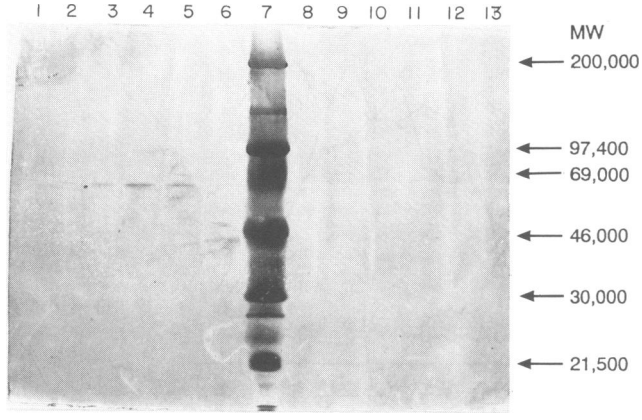

Figure 4. Silver stained SDS–PAGE gel of eluates from ELISA wells incubated with reduced and alkylated Sephadex-activated serum. Lanes 1–6 show activated NHS after 0 min, 10 min, 30 min, 2 hr, 4 hr and 1 week of activation; lane 7, MW markers (same as in Fig. 3), lanes 8–13 factor I-deficient serum (activation time as in lanes 1–6).

intensity pattern as described above (Fig. 3b). The anti-C3c antibody from Dakopatts does not recognize the thiolester-containing 62,500 MW N-terminal fragment of the α-chain, which was, however, visualized with the anti-C3d antibody used in Fig. 3b. The pattern of reactivity of anti-C3c and anti-C3d antibodies (i.e. the failure of anti-C3c to recognize the 63,000 MW iC3b α-chain fragment and the 20,000–25,000 C3c α-chain fragment which corresponds to the N-terminal region of the 63,000 MW fragment) is in agreement with the observation by Folkersen *et al.*[31] The high molecular weight bands developed with the anti-C3d antibody showed the presence of the thiolester-containing C3 fragments bound to other serum proteins.

EDTA eluates samples of activated serum reduced with DTT prior to the incubation with conglutinin on the ELISA plates were also analysed. The SDS–PAGE gel was silver stained (Fig. 4). This approach revealed a band of 63,000 MW after incubation with serum activated for 30 min–4 hr. No bands were observed after prolonged activation (1 day or more). Also, no bands were observed using the factor I-deficient serum (Fig. 4). The interaction between conglutinin and this peptide was calcium dependent and inhibitable with 2 mM GlcNAc (data not shown), demonstrating that the interaction is caused by the lectin-nature of conglutinin.

DISCUSSION

The complement system in human serum was activated and binding of conglutinin to fluid-phase C3 fragments was estimated with anti-C3c and anti-C3d antibodies. Anti-C3c antibody will react with the following C3 components: C3, C3b, iC3b and C3c, while anti-C3d antibody reacts with: C3, C3b, iC3b and C3d (Fig. 1). The ELISA results showed that, at the experimental conditions employed, C3 products with affinity for solid-phase conglutinin became detectable after about 10 min of complement activation (Fig. 2). Clearly, intact C3 was not being bound. No binding was observed in the factor I-deficient or factor I-depleted sera, and the binding was restored by reconstitution with purified factor I. This shows that the generation of reactive fragments is dependent on the presence of factor I, and that C3b has to be processed by factor I to the fragment iC3b before an interaction with conglutinin can occur. The finding of the same kinetics using anti-C3c and anti-C3d antibodies and the inability of the C3 fragments in fully converted serum to interact with conglutinin shows that neither the C3c fragment nor the C3d fragment interacts with conglutinin. Fully converted serum contains no C3, C3b or iC3b but only C3c, C3d and smaller C3 fragments. Thus, from the ELISA results it can be concluded that, when complement activation proceeds in intact serum, conglutinin only interacts with iC3b.

When the Western blot of C3 fragments bound by conglutinin was reacted with anti-C3c, bands common to iC3b and C3c, but not C3 or C3b, were observed with the same complement activation time dependency as observed in the ELISA (Fig. 3a). A band of 75,000 MW was observed, which is a characteristic of the β-chain of C3, and this chain remained unchanged during the activation and inactivation of C3 (Fig. 1). An additional band of 40,000 MW was also observed. This band corresponded to the C-terminal fragment of the α-chain of iC3b (Fig. 1). This polypeptide is also present in C3c. A band of 63,000 MW representing the N-terminal fragment of the α-chain of iC3b was also expected. However, the anti-C3c antibody employed showed no reactivity with epitopes on this N-terminal fragment, consistent with previous analyses.[31] When the blot was developed with an antibody against C3d, a band of 63,000 MW appeared (Fig. 3b). This band represents the N-terminal α-chain fragment of iC3b generated by factor I cleavage of the α-chain of C3b.

The activated serum was reduced and alkylated in order to disrupt the disulphide bonds in C3 before the interaction with conglutinin. The conglutinin-binding material from such serum samples showed only a band of 63,000 MW on a silver-stained SDS–PAGE gel (Fig. 4). The kinetics for the appearance of this band during the activation of serum followed the response seen in the ELISA. This experiment showed that under these conditions conglutinin specifically interacts with the N-terminal fragment of the α-chain of iC3b and that the rest of the molecule need not be associated with this polypeptide in order for adequate oligosaccharide presentation. Interestingly, this demonstrates that the non-covalent interactions between these two parts of the C3 molecule can only be weak. Human C3 is known to possess two high mannose-type oligosaccharides, one at residue 917 on the α-chain, and one at residue 63 on the β-chain. The two high mannose oligosaccharides share the common core structure with α 1–3- and α 1–6-linked mannose units, but the oligosaccharide on the α-chain of C3 has one or two additional linked α 1–2 mannose units.[9,23,32] The interaction of conglutinin only with the oligosaccharide on the α-chain may be attributable to these terminal α 1–2 mannose units.[9,11,23]

During the activation of C3 to C3b and the following proteolytic cleavage to iC3b, C3c and C3d, the oligosaccharides on both the α- and the β-chain supposedly remain unchanged. Therefore, the highly selective interaction of conglutinin with the oligosaccharide on the α-chain of the iC3b fragment is dependent on the conformation of the peptide chain. On the C3 and C3b molecules the oligosaccharide may be shielded by the peptide chain and therefore not accessible for the lectin. It appears that cleavage of C3b to iC3b induces a conformational change resulting in an exposition of the terminal α 1–2 mannosyl residues in a manner suitable for the binding of conglutinin. When iC3b is degraded to C3c the oligosaccharide is again rendered inaccessible to conglutinin. Conformational changes

in C3 upon activation and inactivation have also been found by others using monoclonal anti-C3 antibodies.[33,34] An alternative explanation would be that conglutinin recognizes not only the oligosaccharide but also some surrounding peptide structures available only on the iC3b fragment or that the polypeptide influences the conformation of the oligosaccharide. Exposure of oligosaccharides upon activation of C3 is indicated by the differential susceptibility to endoglycosidases.[9] Endoglycosidase H is able to remove the oligosaccharide from C3b in the absence of detergent, while the enzymatic removal of oligosaccharides from C3 requires prior treatment with SDS,[9] suggesting a steric inaccessibility of the oligosaccharide in native C3.

Conglutinin was previously reported to interact not only with fluid-phase iC3b but also with C3b and, most strongly, with C3c.[23] This is in contrast with earlier reports from several groups dealing with membrane-associated complement components where interaction could only be observed with iC3b.[2,4-6] Hirani *et al.* digested purified C3 with different enzymes to yield C3b, iC3b, C3c and C3d.[23] Their ELISA system for detecting the interaction was essentially the same as the one employed in the present work. It seems possible that purification-induced alteration of the conformation of C3 could be the reason for the observed interactions with C3b and C3c, or the use of purified proteases used may generate fragments stereochemically different from the fragments generated in serum. We therefore replicated the system used by Hirani *et al.*[23] and found that conglutinin interacted with purified C3, C3b and C3c (binding of iC3b was not examined). Hirani *et al.*[23] did not report on the binding of C3.

The discrepancy between binding of fragments generated in serum milieu and those made from purified C3 stresses the importance of investigating biological phenomena at conditions approaching the physiological situation.

We also investigated the binding of two other collectins, mannan-binding protein (MBP) and collectin-43 (CL-43) to C3 and degradation products thereof. These two collectins also reacts with mannose residues, and it was surprising to find that no interaction could be detected between these two collectins and any of the forms of C3.

The remarkably specific interaction of conglutinin with the high mannose-type oligosaccharide on iC3b is to our knowledge the only example so far of a lectin-reactive carbohydrate epitope dependent upon the conformation of the peptide.

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