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Defined chemically cross-linked oligomers of human C-reactive protein: characterization and reactivity with the complement system

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SUMMARY

Chemically cross-linked C-reactive protein (CRP) oligomers were prepared and characterized, and C1q binding and C activation were investigated.[1] Purified human CRP was polymerized in the presence of both non-cleavable and cleavable cross-linking agents and further separated by Superose 12 analytical FPLC column chromatography into fractions of 110 KDa (pentameric monomers), 220 KDa (dimers) and 330 KDa (trimers); virtually no larger oligomers were formed under a variety of experimental conditions. CRP subunits were cross-linked both within and between CRP pentamers. CRP trimers retained native CRP antigenicity without expression of neo-CRP epitopes. CRP trimers showed maximal binding and CRP dimers showed partial binding of solid phase C1q while CRP monomers bound virtually no C1q at all; CRP trimers also bound to fluid phase C1q. Binding was Ca^{++} independent and increased as the ionic strength or pH were lowered, characteristics comparable to binding of aggregated IgG to C1q; it was not inhibited by phosphorylcholine. CRP trimers consumed total C, C1 and C2 haemolytic activities upon incubation in fresh human serum, but much less efficiently than did CRP–protamine complexes or Agg-IgG. CRP trimers failed to deplete alternative C pathway haemolytic activity at all. The stable, chemically defined CRP oligomers described in this report, which bind C1q efficiently but display poor ability to activate the classical C pathway in the absence of an appropriate ligand, should be valuable in further studies of the interactions between CRP and the C system.

INTRODUCTION

C-reactive protein (CRP) is an acute phase serum protein composed of five identical 23-KDa subunits.[2,3] Like IgG, CRP can induce reactions of precipitation and agglutination, and modulate phagocyte function.[4-8] CRP-ligand complexes, like IgG complexes, also react with C1q and activate the classical complement (C) pathway.[9-14] C1q consists of six subunits divided into globular (GR) and collagen-like (CLR) regions. IgG complexes bind to and activate C via the C1q GR[15,16] while CRP complexes bind to and activate C via the C1q CLR.[17] In the absence of aggregation induced by ligands or cross-linking agents, CRP (like IgG) does not interact, or interacts only weakly, with C1q; however, upon binding to a multivalent ligand, CRP gains the ability to bind C1q and activate the C system.[9-14] Ligands may function in CRP-induced C activation merely by aggregating CRP and increasing the affinity of CRP for C1q, since chemical aggregates of CRP have C1q-binding and C-depleting capacity, but additional participation of the ligand or reactive surface in CRP-induced C activation has not been excluded. It is known that heat-aggregated (Agg-IgG) and chemically cross-linked IgG[18-23] bind to C1q and activate haemolytic C in the absence of antigen. By contrast, heat-aggregated CRP lacks ability to activate the C system.[24] However, CRP modified by heating as well as by acidification or urea-chelation (termed 'modified CRP') undergoes structural alterations reflected in loss of native-CRP antigenicity with appearance of neo-CRP antigens,[25,26] and there is evidence that the native CRP conformation is required for activation of the C system.[17] Hence, in order to test the ability of CRP aggregates to react with C in the absence of antigen, CRP oligomers had to be prepared by a process in which native CRP conformation and antigenicity were preserved. In the present study, such CRP oligomers were prepared with both cleavable and non-cleavable cross-linking agents, and after characterization of the oligomers, their ability to bind C1q and activate the C system was defined.

Abbreviations: Agg-IgG, aggregated human IgG; ABTS, 2-2′ azino-*bis*(e-ethylbenzthiazoline-6-sulphonic acid); BS3, *bis*(sulphosuccinimidyl)suberate; C, complement; CLR, collagen-like region of C1q; CRP, C-reactive protein; DTSSP, 3,3′-dithio-*bis*(sulphosuccinimidylpropionate); DTT, dithiothreitol; EA, sheep erythrocytes sensitized with rabbit antibody; GR, globular region of C1q; GVB, veronal-buffered saline with gelatin; PC, phosphorylcholine; TBS, Tris-buffered saline; $VBST^{++}$, veronal-buffered saline with calcium and Tween-20.

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MATERIALS AND METHODS

Reagents

Phosphorylcholine (PC) chloride, protamine sulphate and *Serratia marcescens* lipopolysaccharide were obtained from Sigma Chemical Co. (St Louis, MO). DE-52 ion exchange celluloses were obtained from Whatman Biosystem Inc. (Clifton, NJ). PC-derivatized keyhole limpet haemocyanin (PC-KLH) was prepared by incubating KLH (Sigma Chemical Co.) with paradiazotized phenolphosphorylcholine (Sigma Chemical Co.) as described.[27] Polyclonal anti-human CRP, SAP and IgG were purchased from INCSTAR Corp. (Stillwater, MN).

Preparation of C1q

C1q was isolated at 4° from human plasma by modification of the method of Tenner *et al.*[28] Briefly, plasma was brought to a final concentration of 5 mM EDTA and applied to a Bio-Rex 70 column (Bio-Rad Laboratories, Richmond, CA), and eluted with a linear (0·08–0·3 M) NaCl gradient; the protein peak was pooled and further purified by gel filtration on a 1·6 × 45 cm preparative Superose 12 FPLC column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), equilibrated, and eluted with buffer consisting of 0·5 M NaCl, 1 mM EDTA and 50 mM Tris at a flow rate of 0·25 ml/min. The absorbance of the eluate at 280 nm was monitored continuously, and the C1q peak was pooled and concentrated using an Amicon concentrator with a PM 30 membrane. The concentration of C1q was determined by its absorbance at 280 nm with use of $E^{1\%}_{1cm} = 6{\cdot}82$.[15] The purity of the final C1q preparation was more than 99 per cent as analysed by SDS–PAGE with silver staining both under unreduced and DTT-reduced conditions. The purified protein was stored at −70°.

Preparation of aggregated human IgG (Agg-IgG)

Human IgG (Gamastan) was purchased from Miles Laboratories (Elkhart, IN) and further purified by FPLC Superose 12 preparative column chromatography as described above in the presence of 10 mM TBS. To prepare Agg-IgG, IgG (7·5 mg/ml) was heated at 63° for 20 min immediately prior to use.

Preparation of CRP

CRP was purified at 4° from filtered human ascites and pleural fluids with modifications of the method described by Potempa *et al.*[25] involving sequential PC affinity chromatography with chelation-elution, anion (DE-52) exchange chromatography, gel filtration chromatography, and repeat PC-affinity chromatography with PC-elution.

Preparation of cross-linked CRP trimers, dimers and monomers

CRP was cross-linked using *bis*(sulphosuccinimidyl) suberate (BS3) or 3,3′-dithis(sulphosuccinimidyl-propionate) (DTSSP) obtained from Pierce Chemical Co. (Rockford, IL).[29] Both reagents (0·1 ml), prepared at 10 mM and dissolved in 10 mM VBS (pH 7·3), were added to 2 ml CRP (1 mg/ml) and incubated at room temperature (RT) for 1 hr; 0·4 ml of 0·1 M TBS was added to stop the reaction. The mixture was centrifuged, and the supernatant was applied to a FPLC Superose 12 analytical column, equilibrated and eluted with 10 mM TBS; gel filtration standard (Bio-Rad Laboratories) containing thyroglobulin (670 KDa), immunoglobulin G (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa) and vitamin B12 (1·35 KDa) was used for molecular mass calibration of the column. The first (330 KDa), second (220 KDa) and third (110 KDa) peaks, consisting of trimers, dimers and monomers of CRP, respectively (Fig. 1), were collected and stored at 4°.

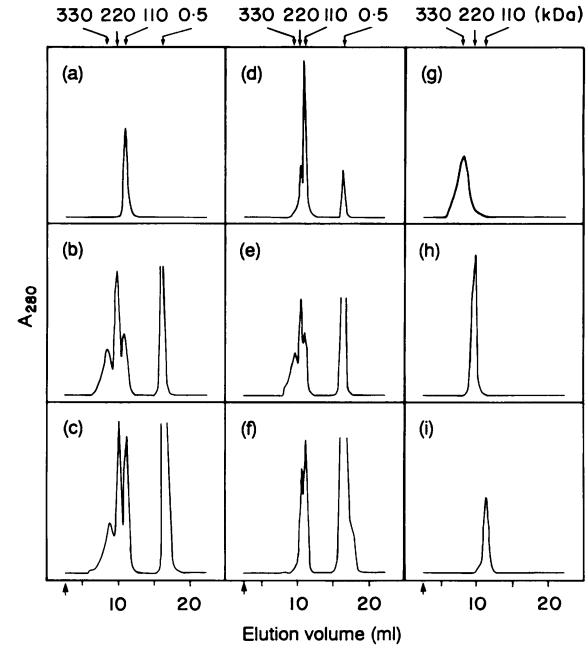

Figure 1. Fast protein liquid chromatographic (FPLC) separation of (a) native CRP, (b) CRP cross-linked with BS3 and (c) CRP cross-linked with DTSSP. The sample (200 μl at 1 mg/ml) was applied to a Superose 12 analytical column, flow rate 0·25 ml/min, in 10 mM TBS containing 2 mM Ca^{++}, pH 7·4. The ratios of cross-linker to CRP were (d) 1:1, (e) 5:1 and (f) 20:1. BS3 cross-linked CRP was further purified as (g), trimers, (h) dimers and (i) monomers by three cycles of FPLC.

SDS–PAGE analysis

SDS–PAGE was carried out on 12·5% polyacrylamide minislab gels (Bio-Rad Laboratories) using the buffer system described by Laemmli.[30] After SDS–PAGE, the protein bands were identified by silver stain using kits obtained from Bio-Rad Laboratories.

Biotinylation of CRP and IgG

Purified human CRP trimers, dimers, monomers and Agg-IgG each were dialysed into 0·1 M sodium bicarbonate buffer (pH 9·0), and incubated with NHS-LC Biotin (Pierce Chemical Co.) at ratios of 8:1 (w:w, protein:biotin) for 4 hr at room temperature. The mixtures were dialysed twice on a magnetic stirrer against 4 litres of 10 mM TBS^{++} at 4°.

Solid-phase ligand-binding assays

Wells of microtitre plates (C. A. Greiner and Sohne, Nurtingem, Germany) were coated overnight at 4° with 100 μl per well C1q or KLH-PC (5–10 μg/ml), backcoated with BSA (200 μl of a 1% solution), and incubated at 37° for 30 min. The plates were washed with VBS^{++}, and in most experiments biotinylated CRP oligomers were added (37°, 30 min) followed by washing, addition of Streptavidin-peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) and incubation of the plates for another 30 min at 37°; in KLH-PC binding experiments non-biotinylated CRP oligomers were used and detected with goat

anti-human CRP (INCSTAR Corp.) followed by rabbit anti-goat peroxidase (Jackson Immunoresearch Laboratories). The plates were washed, substrate (ABTS in 0·1 M sodium citrate at pH 4·0) (Bio-Rad Laboratories) was applied and reactions were allowed to develop at 37° for 10–30 min. The $A_{(414)}$ nm was read in a Titertek Multiskan MC plate reader.

Total C (CH_{50}) consumption

Human serum was obtained from normal laboratory volunteers and absorbed with PC-beads at room temperature for 30 min to remove CRP and anti-PC antibody. The CH_{50} was determined by a modification[11] of the method of Mayer.[31] A mixture of 0·1 ml of 1:10 human serum and 0·1 ml test material (CRP trimers, CRP–protamine complexes, or Agg-IgG) was incubated for 30 min at 37°; twofold dilutions were prepared, 0·1 ml erythrocytes (E) sensitized with haemolysin (1×10^8 EA/ml) were added and the mixture was incubated for 30 min at 37°. GVB-EDTA (1 ml) was added, the solution was centrifuged for 10 min at 1100 *g*, the optical density of the supernatant was measured at 412 nm and the per cent C consumed was calculated in the usual way.[32]

Alternative pathway C consumption

A mixture of 0·1 ml of 1:2 normal human serum in VBS-EGTA-Mg^{++} and 0·1 ml test material (CRP trimers, CRP monomers, IgG or endotoxin) was incubated for 15 min at 37°, twofold dilutions were prepared, 0·1 ml rabbit E (1×10^8) was added and the mixtures were incubated for 60 min at 37°. Cold GVB-EDTA (1 ml) was added, and after centrifugation (10 min) at 2000 r.p.m., the optical density of the supernatant was measured at 412 nm; the per cent C consumed was calculated in the usual way.[32]

Haemolytic C component assays

C1, C4 and C2 haemolytic activities were measured exactly as described.[12,14,33]

C components and C-deficient sera

C1 and C2 were prepared from guinea-pig serum as described by Nelson *et al.*[34] C1q-depleted human serum was obtained from Sigma Chemical Co. C4-deficient guinea-pig serum was the kind gift of Dr Alexander P. Osmand (Oak Ridge Laboratories, Knoxville, TN).

Monoclonal antibodies (mAb) to CRP

Murine mAb to human CRP were purified from ascites fluids as previously described.[26]

RESULTS

Preparation of cross-linked CRP oligomers utilizing BS3 and DTSSP.

CRP (1 mg/ml) was cross-linked with BS3 (1:5–10, M:M) and the mixture was separated by fast protein liquid chromatography (FPLC) gel filtration (Fig. 1b) as trimers (330 KDa), dimers (220 KDa) and monomers (110 KDa); a fourth peak consisting of the free BS3 cross-linker (0·57 KDa) also was obtained. Under these conditions cross-linking yielded approximately 15 per cent trimers, 50 per cent dimers and 35 per cent monomers; no protein (<2·0%) of larger size was detected.

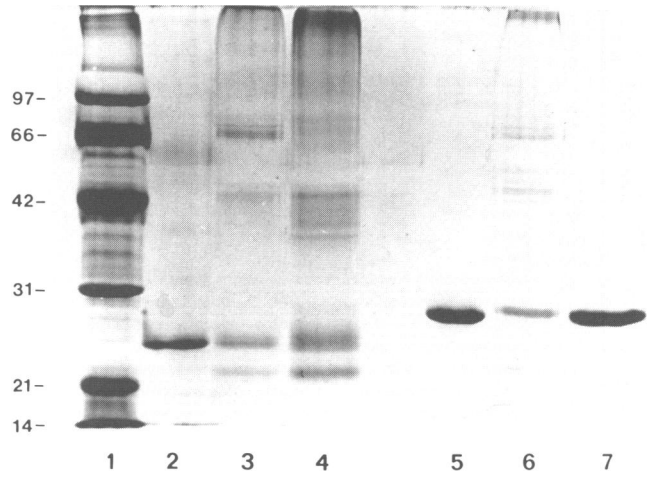

Figure 2. SDS–PAGE (12·5%) were performed under non-reducing (lanes 2–4) and reducing (lanes 5–7) conditions, with protein was detected by silver stain. CRP (lanes 2 and 5), BS3 cross-linked CRP trimers (lanes 3 and 6) and DTSSP cross-linked CRP trimers (lanes 4 and 7. Molecular weight standards are shown in lane 1.

Identical results were obtained when the cleavable cross-linker DTSSP, which like BS3 cross-links at lysine residues, was used (Fig. 1c). The optimal ratio of BS3 to CRP was approximately 5–10:1 (M:M; Fig. 1e). At lower (1:1; Fig. 1d) or higher (20:1; Fig. 1f) ratios of cross-linker:CRP, only dimers were formed but no trimers. Increasing the concentration of CRP (to 6 mg/ml) did not increase the yield of CRP trimers, nor were oligomers larger than trimers observed. CRP trimers, dimers and monomers were further purified to homogeneity by three cycles of FPLC (Fig. 1g, h) The purified CRP trimers obtained represented approximately 10% of the CRP exposed to BS3, and were stable as trimers for at least 6 months as assessed by repeat analytic FPLC.

Chemical characterization of CRP oligomers

The CRP oligomers first were characterized by SDS–PAGE. The migratory band of CRP subunits under non-reducing (lane 2) and reducing conditions (lane 5) are shown in Fig. 2. CRP migrated more slowly after treatment with DTT because of reduction of the intra-subunit disulphide bond. CRP trimers cross-linked by BS3 (lane 3) and DTSSP (lane 4) also showed a faster-moving band which disappeared under reducing conditions (lane 7), indicating cross-linking within subunits of pentameric CRP rather than fragmentation, perhaps with formation of 'supercoiled' CRP subunits, had occurred. Cross-linking between subunits of different pentamers, including two-, three-, and four-subunit bands which coalesced into a smear, also was observed. In addition, material >150 kDa which failed to enter the unreduced gels, in all likelihood CRP trimers, were seen in lanes 2, 3, 4 and 6. Following reduction and alkylation, the CRP trimers formed with the non-cleavable aggregating agent BS3 retained a migration pattern identical to that observed in SDS alone (lane 6); by contrast, after reduction and alkylation CRP trimers formed with the cleavable aggregating agent DTSSP migrated with a size identical to that of native CRP subunits exclusively (lane 7), reflecting disruption of the bonds formed by the cross-linker.

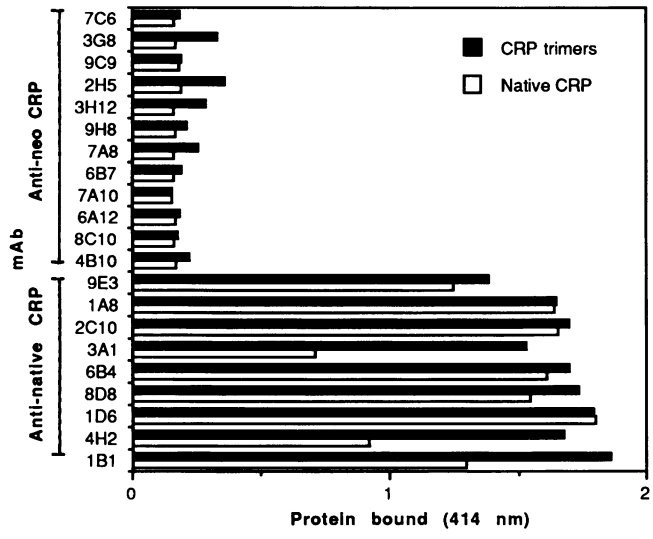

Figure 3. CRP and CRP trimers (0·5 μg/well) captured by KLH-PC (0·5 μg/well) immobilized on polystyrene plates during 30-min incubations at 37°; anti-native and anti-neo CRP mAb (20 μg/ml; 100 μl/well) were used to detect CRP; the reactions were developed with goat anti-mouse-peroxidase and substrate.

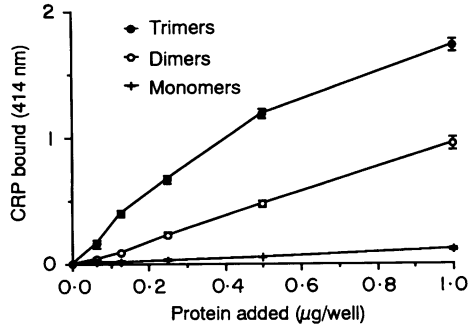

Figure. 4. Binding of cross-linked (with BS3) biotinylated CRP trimers, dimers and monomers (0–10 μg/ml) to C1q (0·5 μg/well) immobilized on polystyrene plates during 30-min incubations at 37°; the reactions were developed with Streptavidin-peroxidase and substrate.

Antigenic characterization of CRP oligomers

The CRP trimer preparation retained ability to bind PC, as determined by complete absorbtion to PC-Sepharose in the presence of calcium and elution in the presence of citrate (data not shown). The antigenicity of CRP trimers captured by immobilized PC-KLH is shown in Fig. 3. CRP trimers, like native CRP, expressed exclusively native epitopes, which were detected by each of 9 mAb specific for native CRP. There was no reactivity with any of 12 mAb specific for neo-CRP epitopes.

Binding of cross-linked CRP to C1q

The binding to immobilized C1q of CRP trimers and dimers cross-linked with BS3, as well as CRP monomers, was compared. CRP trimers had the highest binding activity on a weight basis; CRP dimers also bound C1q, while CRP monomers displayed little or no C1q binding at all (Fig. 4). CRP cross-linked with DTSSP showed comparable binding of C1q. The

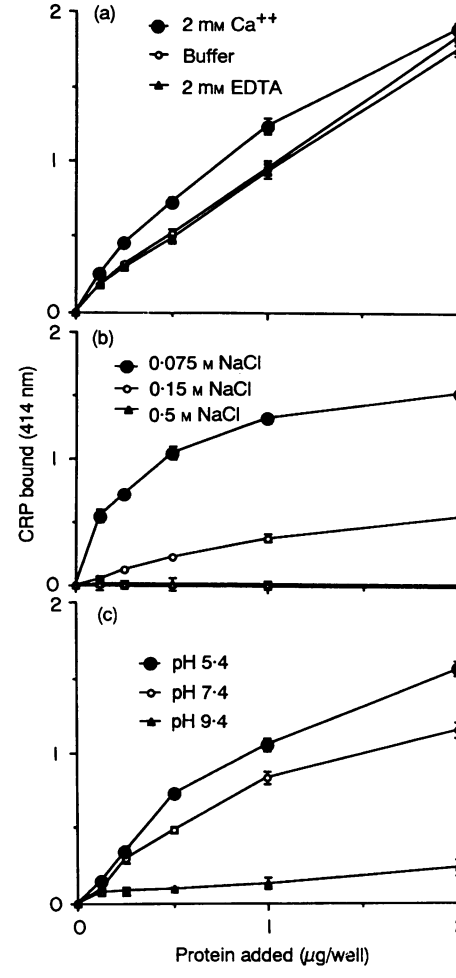

Figure 5. Binding of biotinylated CRP trimers (0–20 μg/ml) to immobilized C1q (0·5 μg/well) in buffer in the presence or absence of either 2 mM Ca^{++} or 2 mM EDTA is shown in (a); binding of CRP trimers (0–20 μg/ml) to C1q (0·5 μg/well) at increasing ionic strength (0·075, 0·15 and 0·5 M NaCl) is shown in (b); and binding of CRP trimers (0–0·20 μg/ml) to C1q (0·5 μg/well) at increasing pH (5·4, 7·4 and 9·4) is shown in (c).

binding of CRP trimers to immobilized C1q was inhibited by preincubation with fluid phase C1q; 50 μg/ml C1q inhibited CRP binding by 50 per cent.

Effect of Ca^{++}, pH and ionic strength on the interaction of CRP trimers with C1q

CRP trimers bound to C1q in the presence of 2 mM EDTA, although somewhat greater binding was observed in 2 mM Ca^{++} (Fig. 5a). Binding increased as the ionic strength was lowered (Fig. 5b). Low pH (5·4) enhanced binding of CRP trimers, while binding was lost at high (9·4) pH (Fig. 5c). The binding of CRP trimers to C1q was similar to that of IgG under all of these conditions.

CRP trimers induce consumption of total C, C1 and C2 haemolytic activities

Incubation of CRP trimers in normal human serum at 37° resulted in depletion of classical pathway total haemolytic C

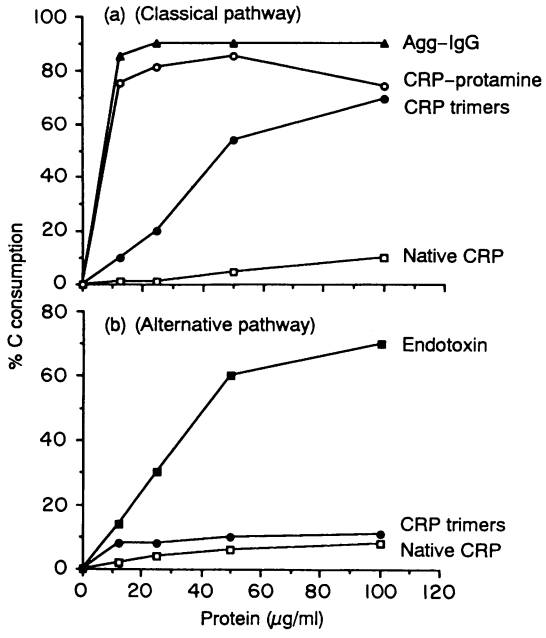

Figure 6. Total classical pathway haemolytic C consumption induced by increasing concentrations of CRP trimers (●) compared to CRP–protamine complexes (○), native CRP (□) and Agg-IgG (▲) in normal human serum absorbed with PC beads (a), with absence of alternative pathway haemolytic C consumption by CRP trimers (●) as compared to endotoxin (■) and native CRP (□) (b).

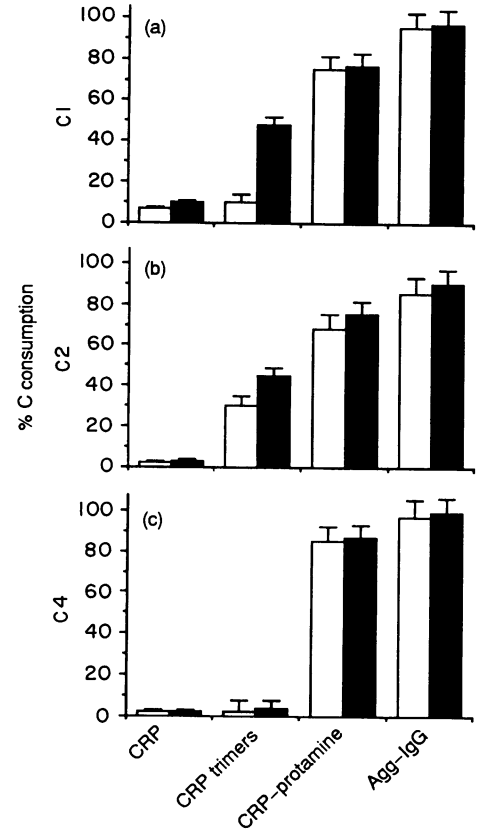

Figure 7. Haemolytic C1, C4 and C2 consumption induced by 50 (open bars) or 100 μg/ml (closed bars) CRP trimers, as compared to CRP–protamine complexes (expressed as μg CRP), native CRP (expressed as μg CRP) and Agg-IgG (expressed as μg protein), upon preincubation in normal human serum. Titres of C1, C4 and C2 were 100,000, 200,000 and 200 haemolytic units, respectively.

activity (CH_{50}), although significantly greater amounts (10-fold; approximately 50 μg/ml) were required than for 50% C depletion by CRP–protamine complexes (5 μg/ml; Fig. 6a). C1 and C2 haemolytic activities were consumed, but again, much greater amounts of CRP trimers, than CRP–ligand complexes, were required (Fig. 7); no C4 consumption was detected. Addition of protamine to CRP trimers, like addition of protamine to native CRP, resulted in complete depletion of C1, C4 and C2. CRP trimers lacked ability to deplete alternative pathway haemolytic C activity (Fig. 6b).

DISCUSSION

Heat-aggregated and chemically cross-linked IgG bind to C1q and activate the C system in the absence of antigen.[19 23,35] Like IgG, ligand-complexed CRP can react with C1q and activate the classical C pathway.[9,11] However, in contrast to heat-aggregated IgG, heat-aggregated CRP lacks ability to activate the C system.[24] This is consistent with the hypothesis that a conformational rather than a sequence-determined site is involved in C1q binding and C activation by CRP, an observation supported by the ability of an anti-CRP mAb (8D8) directed against a conformational determinant to inhibit the ability of CRP–ligand complexes to bind C1q and activate the C system.[17] Recent investigations showed that chemically cross-linked CRP possessed ability to bind C1q and consume haemolytic C activity,[1] but did not address whether these oligomers had the ability to activate the C system. The present studies were initiated to prepare optimally reactive CRP oligomers, characterize the oligomers chemically and antigenically, and more fully define their interactions with C1q and the complement system.

For this purpose, oligomers of human CRP were prepared by use of the bifunctional, non-cleavable cross-linking agent BS3, which binds to lysine residues, and purified to homogeneity utilizing FPLC. The optimal ratio of cross-linker to CRP subunits was about 5–10:1. Oligomers were also prepared with the cleavable cross-linker, DTSSP, which like BS3 reacts with lysine groups, and this agent displayed a virtually identical capacity to cross-link CRP. The largest soluble CRP aggregates obtained with either cross-linker were trimers. The reason for lack of formation of larger aggregates is unknown, but increasing the concentration either of cross-linker or of CRP did not increase the yield or size of CRP oligomers. FPLC and SDS-PAGE analyses suggested that three types of cross-linking had occurred, involving bonds within subunits, between subunits of the same pentamer and between subunits of different pentamers.

CRP trimers prepared with either cross-linker were easily biotinylated and stable for at least 6 months at 4°. Native CRP immunoreactivity was maintained without expression of neo-CRP epitopes as determined by full reactivity with each of nine anti-native-CRP mAb in the absence of reactivity with any of 12 anti-neo-CRP mAb. These data are important since C activation had been observed preferentially with CRP–ligand complexes in which the native CRP conformation and antigenicity were maintained, while no C activation was observed with

heat-induced CRP aggregates in which native CRP conformation and antigenicity were lost.[24]

The interaction of CRP trimers with C1q allowed characterization of the interaction of CRP with C1q. Similar to IgG aggregates, CRP trimers bound to both immobilized and fluid phase C1q. CRP binding to C1q increased as the ionic strength and pH were decreased, comparable to observations with IgG aggregatres. The increased binding of CRP to C1q at low pH might explain, at least in part, the recent report that CRP activates C in the absence of ligands under mildly acidic conditions.[36]

It has been suggested that the PC-binding site of CRP is involved in C activation.[36] However, the data presented herein indicate the PC-binding site and the C1q-binding site are distinct, since preincubation of CRP trimers with 50 mM PC (amounts considerably in excess of those which completely inhibit binding of CRP trimers to KLH-PC) had no effect on C1q binding, and CRP trimers bound to C1q in the absence of Ca^{++}. The inability of anti-CRP mAb directed against the PC-binding site to block the binding of CRP trimers to C1q[17] provides additional support for the concept that, as for the immunoglobulins, the ligand (PC)-binding site is distinct from the site involved in binding of C1q.

Data presented in this paper demonstrate that CRP trimers bind C1q with minimal but distinct activation of the classical C pathway. Much greater amounts of CRP trimers than CRP–ligand complexes were required to induce consumption of total classical C pathway haemolytic activity, and much less consumption of C components was observed. C1 and C2 were consumed in the apparent absence of consumption of C4, although in analogy to complement consumption by zymosan in the original investigations of the properdin system (reviewed in ref. 29), this may simply reflect the greater amounts of C4 as compared to C2 present in human serum. Thus, while CRP–ligand complexes and CRP trimers bind comparably to the C1q CLR,[17] the former induce significantly greater activation of the classical C pathway. These data suggest a requirement additional to C1q binding for CRP-induced C activation, perhaps involving a ligand–C1 subcomponent or ligand–control protein interaction or an additional conformational change of CRP. Thus, in analogy to activation of the alternative C pathway,[37] the ligand or surface involved in CRP binding may play a role in CRP-ligand C activation. Evidence for requirement of a second signal, in addition to binding of C1q, to achieve C1 activation generally has been reviewed by Cooper[38] and this may be particularly relevant for agents which, like CRP, react with the C1q CLR. In any case, the use of cross-linked CRP, as described in the present paper, should be valuable in studying the interaction not only between CRP and the C system, but also interactions between CRP and formed elements and proteins of the blood which occur preferentially when this pentraxin is in the aggregated or complexed state.

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