Inhibition of the Reconstitution of the Haemolytic Activity of the First Component of Human Complement by a Pepsin-Derived Fragment of Subcomponent C1q

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1. A fragment of subcomponent C1q, which contained all the collagen-like features present in the intact molecule, was isolated by pepsin digestion as described by Reid [*Biochem. J.* (1976) 155, 5–17]. 2. The pepsin-derived fragment of subcomponent C1q did not bind to antibody-coated erythrocytes under conditions where complete binding of subcomponent C1q took place. 3. The peptic fragment blocked the reconstitution of C1 haemolytic activity by competing with intact subcomponent C1q in the utilization of a mixture of the other two subcomponents, C1r and C1s. 4. Reduction and alkylation of the interchain disulphide bonds in the pepsin fragment did not markedly affect its inhibitory effect, whereas heating at 56°C for 30min completely abolished the effect. 5. Lathyrin rat skin collagen and CNBr-derived peptides of pig type II collagen showed no ability to mimic the inhibitory effect of the pepsin fragment when tested over the same concentration range as used for the peptic fragment. 6. The peptic fragment was unable to block efficiently the reconstitution of C1 haemolytic activity unless it was added to the mixture of subcomponents C1r and C1s before the attempt to reconstitute C1 haemolytic activity, in solution, or on the surface of antibody-coated erythrocytes. 7. Evidence was obtained that suggested that subcomponent C1q bound the subcomponent C1r–C1s complex more efficiently when the subcomponent C1q was bound to antibody than when it was free in solution.

The first component of complement is composed of three subcomponents, C1q, C1r and C1s, and reconstitution of intact C1 haemolytic activity can be achieved by mixing the three separated components together in the presence of Ca^{2+} ions (Lepow *et al.*, 1963; Gigli *et al.*, 1976). Subcomponent C1q has two distinct roles to play in the activation of the classical pathway of complement. The first is a recognition and binding role that involves the binding of subcomponent C1q to immune complexes involving immunoglobulins of the IgG* and IgM classes via the Fc regions in these molecules (Tranta & Franklin, 1961; Ishizaka *et al.*, 1966; Augener *et al.*, 1971). The second is an activation role during which subcomponent C1r is converted from a proenzyme into an enzyme, by the splitting of probably a single peptide bond (Takahashi *et al.*, 1975; Ziccardi & Cooper, 1976a; Sim & Porter, 1976), which allows activation of the subcomponent C1s proenzyme to yield the fully active C1 complex. The internal activation of subcomponent C1r in the C1 complex could possibly be brought about by a conformational change taking

place in the subcomponent C1q molecule on its binding to immune complexes, since no enzymic activity has so far been detected in subcomponent C1q that could be responsible for the activation of proenzymic subcomponent C1r.

The subcomponent C1q molecule, as shown in the electron microscope, consists of six peripheral globular regions each joined by a connecting strand to a fibril-like central portion (Shelton *et al.*, 1972; Knobel *et al.*, 1975), and a molecular model, in which most of the six connecting strands and fibril-like central portion are considered to be composed of collagen-like triple-helical structure, has been proposed (Reid & Porter, 1976). There is some evidence that the peripheral globular regions, which are resistant to collagenase digestion (Reid *et al.*, 1972) but which are readily digested by pepsin (Reid, 1976), may contain binding sites for IgG (Knobel *et al.*, 1974). In this present paper the pepsin-derived fragment of subcomponent C1q, which contains all the collagen-like features present in the intact molecule (Reid, 1976; Brodsky-Doyle *et al.*, 1976), was examined to test whether it retained any of the properties of the intact molecule.

* Abbreviations: IgG and IgM, immunoglobulin G and immunoglobulin M (macroglobulin) respectively.

Materials and Methods

Isolation of subcomponent C1q, the peptic fragment of subcomponent C1q and the proenzymic forms of subcomponents C1r and C1s

Subcomponent C1q was isolated as described by Reid (1974). The peptic fragment was isolated as described by Reid (1976) and reduced and alkylated as described by Brodsky-Doyle *et al.* (1976). Stock solutions of subcomponent C1q and of the pepsin fragment were made, at a concentration of 1 mg/ml, in the veronal-buffered saline used in the haemolytic assays and stored at -20°C . The $A_{1\text{cm}}^{1\%}$ at 280 nm of human subcomponent C1q was taken as 6.82 and its mol.wt. taken as 410000 (Reid *et al.*, 1972). The concentration of peptic fragment was determined by amino acid analysis and its mol.wt. was taken to be 176000 (Reid, 1976).

Subcomponents C1r and C1s were isolated in their proenzymic forms essentially as described by Gigli *et al.* (1976). A stock solution of 0.44 mg of protein/ml, composed of equimolar amounts of subcomponents C1r and C1s, was stored at 4°C . The $A_{1\text{cm}}^{1\%}$ values at 280 nm of subcomponents C1r and C1s were taken as 11.5 and 9.5 respectively (Gigli *et al.*, 1976) and the mol.wts. of each taken as 83000 (Sim & Porter, 1976).

Buffers used in complement assays

Veronal-buffered saline, containing 73 mM-NaCl, 0.9 mM-sodium barbitol, 1.56 mM-barbitol, 0.15 mM- CaCl_2 , 0.5 mM- MgCl_2 , 0.05% (w/v) gelatin and 2.5% (w/v) D-glucose, pH 7.5, was used in all the haemolytic assays. EDTA buffer, pH 7.4, was made by making a 1:2.5 dilution of a stock solution of 0.1 M-EDTA, pH 7.4, with iso-osmotic veronal-buffered saline containing 0.05% (w/v) gelatin.

Complement components and cellular intermediates

Functionally pure guinea-pig component C2 was prepared as described by Nelson *et al.* (1966), and C-EDTA was made by diluting guinea-pig serum 1:20 with EDTA buffer, pH 7.4. EAC4 cells were made by the method of Borsos & Rapp (1967). EAC1q4 cells were prepared by mixing EAC4 cells (1×10^8 /ml) with an equal volume of a solution of human subcomponent C1q (0.650 pmol/ml), and incubating the mixture for 30 min at 30°C and then washing the EAC1q4 cells thus formed, twice, at 4°C , and finally resuspending them at a concentration of 1×10^3 cells/ml. Veronal-buffered saline as used in the assays was used to prepare the EAC1q4 cells.

Reconstitution of C1 haemolytic activity

The reconstitution of C1 haemolytic activity was performed in two ways. The first was in solution, with the use of EAC4 cells to monitor the amount of

C1 formed; the second was on the surface of the erythrocyte by adding subcomponents C1r and C1s to EAC1q4 cells.

To reconstitute C1 haemolytic activity in solution, subcomponent C1q in the veronal-buffered saline assay medium was added to a mixture of subcomponents C1r and C1s, also in veronal-buffered saline assay medium (the proportions of subcomponents being 1:2:2 for C1q/C1r/C1s). The mixture was incubated at 30°C for 15 min, then EAC4 cells (0.5 ml, 1×10^8 cells/ml) were added to a dilution of the reconstituted C1 and the mixture was incubated at 30°C for 20 min. The EAC14 cells thus formed were washed once with the veronal-buffered saline assay medium (1.0 ml) at 4°C and then resuspended in veronal-buffered saline assay medium (1.0 ml) and incubated at 30°C for 60 min. Then component C2 (0.5 ml), in veronal-buffered saline assay medium, was added and the mixture was incubated at 30°C for 15 min. EDTA buffer (0.5 ml), pH 7.4, was then added, and finally C-EDTA (1.0 ml) was added and incubation continued for 1 h at 37°C . The degree of lysis was determined by reading the $A_{1\text{cm}}^{412\text{nm}}$ of the 1000g supernatant, after addition of 4.5 ml of 0.15-M NaCl.

To reconstitute component C1 haemolytic activity, on the cell surface, EAC1q4 cells were added to a dilution of subcomponents C1r and C1s and the mixture was incubated at 30°C for 15 min. Then the EAC14 cells thus formed were treated in exactly the same way as described above.

Inhibition of the reconstitution of C1 haemolytic activity

In experiments where the inhibition of the reconstitution of C1 haemolytic activity in solution was studied, by using EAC4 cells, the following procedure was used, the mixture of subcomponents C1r and C1s (0.1 ml) in veronal-buffered saline assay medium was added to the inhibitor (0.1 ml of a dilution of the stock solution), also in veronal-buffered saline assay medium, and the mixture was incubated at 30°C for 15 min. Subcomponent C1q (0.2 ml) was then added in veronal-buffered saline assay medium, and, after a further incubation at 30°C for 15 min, a dilution of the mixture was assayed, as described above, after addition of EAC4 cells. The degree of inhibition in the tubes containing the pepsin fragment was estimated by comparing the degree of lysis in these tubes with that obtained in control tubes which contained a mixture of the subcomponents C1q, C1r and C1s but no pepsin fragment. This inhibition assay was performed, as described above, in an attempt to study the interference, by the peptic fragment, with the reconstitution of the C1 molecule free in solution rather than by binding on to the cell surface, since all three subcomponents were preincubated with the inhibitor before addition of the EAC4 cells.

Fig. 1. Inhibition of the reconstitution of C1 haemolytic activity, in solution, by the peptic fragment of subcomponent C1q

Constant amounts of the mixture of subcomponents C1r and C1s were incubated with increasing concentrations of the peptic fragment before addition of a constant amount of subcomponent C1q and the assay of C1 haemolytic activity formed by using EAC4 cells. There was 100% lysis in the absence of the peptic fragment, and subcomponents C1q, C1r and C1s were always in the proportions 1:2:2 in the final mixture.

In experiments where the inhibition of the reconstitution of C1 haemolytic activity was studied with EAC1q4 cells the procedure used was identical with that used for EAC4 cells, except that the inhibitor (0.25 ml) was added to a mixture of subcomponents C1r and C1s (0.25 ml), and the mixture was incubated at 30°C for 15 min, then EAC1q4 cells were added directly to the mixture without dilution of the mixture. This assay procedure therefore allows study of the reconstitution of C1 haemolytic activity on the cell surface, with the use of low concentrations of reagents relative to those used in the C1 reconstitution in solution, and also excludes the possibility that any inhibition of the reconstitution of C1 haemolytic activity observed is due to interference with the binding of subcomponent C1q to antibody.

In several experiments, as discussed below, the order of addition of the solutions of inhibitor, subcomponent C1q, the mixture of subcomponents C1r and C1s and the test cells was changed to see if the peptic fragment was effective at blocking haemolytic activity after allowing fluid-phase assembly, or cell-bound assembly, of component C1.

Isolation of lathyritic rat skin collagen and CNBr-derived peptides of porcine type II collagen

Lathyritic rat skin collagen was obtained by neutral-salt extraction of lathyritic rat skins (Piez *et al.*, 1963) and was provided by Dr. B. Brodsky. The mixture of CNBr-derived peptides of pig type II

collagen were prepared as described by Eyre & Muir (1975) and was provided by Dr. D. R. Eyre.

Results

Inhibition of the reconstitution of C1 haemolytic activity in solution

The peptic fragment of subcomponent C1q was effective in blocking the reconstitution of C1 haemolytic activity in solution when added to the mixture of subcomponents C1r and C1s before the addition of subcomponent C1q (Fig. 1). The C1 reconstitution in solution was normally performed at relatively high concentrations of the subcomponents (20–50 µg/ml), in molar proportions C1q/C1r/C1s 1:2:2, and the addition of and incubation with, the peptic fragment after allowance of the formation of component C1 to take place never gave more than 10% inhibition of the C1 reconstitution as measured on EAC4 cells by using the assay procedure described in the Materials and Methods section. However, when attempts were made to reconstitute C1 haemolytic activity in solution at relatively low concentrations of the subcomponents (0.2–0.5 µg/ml), there was of the order of 80% inhibition, even when the peptic fragment was added after the subcomponents C1q, C1r, and C1s had been incubated together. The molar ratios of the peptic fragment to the subcomponents C1r and C1s used were those that gave no inhibition in the reconstitution experiments performed with the high concentrations of the subcomponents and addition of peptic fragment after the subcomponents had been incubated together. This indicated that component C1 was not efficiently formed, in solution, at these low concentrations, which is in contrast with the formation of C1 haemolytic activity on the surface of EAC1q4 cells (see below). The study of the inhibition of the reconstitution of C1 haemolytic activity in solution by the use of EAC4 cells did not show whether the peptic fragment was interfering with the association of subcomponents C1q, C1r and C1s or with the binding of the reconstituted C1, via subcomponent C1q, to antibody on the EAC4 cells. This being the case, all subsequent experiments were performed with EAC1q4 cells, thus excluding the possibility that any inhibition observed might be due to impairment of C1 binding.

Inhibition of the reconstitution of C1 haemolytic activity on EAC1q4 cells

The peptic fragment was very effective at inhibiting the reconstitution of C1 haemolytic activity at the cell surface of EAC1q4 cells (Fig. 2). The inhibition was found to be highly dependent on the order of addition of reagents and inhibitor, since, if the mixture of subcomponents C1r and C1s was added to the EAC1q4

Fig. 2. Inhibition of the reconstitution of C1 haemolytic activity on EAC1q4 cells by the peptic fragment

A constant amount of the mixture of subcomponents C1r and C1s (0.332 pmol of each/ml) was inhibited from reconstituting C1 haemolytic activity on EAC1q4 cells by increasing concentrations of the peptic fragment. There was 100% lysis of the cells in the absence of peptic fragment. Order of addition of reagents and inhibitor was: ○, the mixture of subcomponents C1r and C1s plus peptic fragment were incubated, then EAC1q4 cells were added; □, a mixture of subcomponents C1r and C1s plus EAC1q4 cells was incubated, then the peptic fragment was added. Experimental details of the full haemolytic assay procedure are given in the text.

Fig. 3. Inhibition of the reconstitution of C1 haemolytic activity on EAC1q4 cells by the peptic fragment with various concentrations of the C1r-C1s subcomponent mixture

Increasing concentrations of the peptic fragment were incubated with three different dilutions of the stock solution of the subcomponent C1r-C1s mixture, before the addition of EAC1q4 cells. □, ○ and △ denote concentrations of 0.332 pmol/ml, 0.664 pmol/ml and 1.328 pmol/ml for the subcomponent C1r-C1s mixture respectively; at these dilutions there was 93, 98 and 100% component C1-dependent lysis of the cells when the peptic fragment was omitted. The percentage degree of inhibition was measured relative to these control values.

cells before addition of peptic fragment, there was no inhibition of activity even at a molar ratio of peptic fragment to subcomponent C1r-C1s mixture of 800:1, whereas a molar ratio of 8:1 gave 43% inhibition when the peptic fragment was added to the subcomponent C1r-C1s mixture before addition of the EAC1q4 cells (Fig. 2).

Since the concentrations of the subcomponent C1r-C1s mixture and subcomponent C1q (on the EAC1q4 cells) are both kept constant in the previous experiment (Fig. 2), it could be argued that inhibition might be due to the peptic fragment interacting with cell-bound subcomponent C1q, rather than interacting with the subcomponent C1r-C1s mixture. The effect of increasing concentrations of the subcomponent C1r-C1s mixture, in the presence of a constant amount of subcomponent C1q, was examined. It can be seen that at high concentrations of subcomponents C1r and C1s there was little or no inhibition of the reconstitution of C1 haemolytic activity on the cell surface, whereas at lower concentrations there was marked inhibition (Fig. 3).

Demonstration that the peptic fragment was inhibiting the reconstitution of C1 haemolytic activity rather than interfering with C1 or subcomponent C1q binding

In addition to the experiments above, which suggest that the peptic fragment was interfering with the association of subcomponents C1r and C1s with subcomponent C1q, the following experiment was performed to investigate this point further: EAC4 or EAC1q4 cells were incubated with the peptic fragment and then spun and washed before incubation with C1, or subcomponents C1r and C1s, and when subsequently assayed for C1 activity no inhibition was observed; moreover, when the supernatant, obtained after incubation of the EAC4 or EAC1q4 cells with peptic fragment, was assayed for inhibitory activity, it was found to contain all the inhibitory activity attributable to the amount of peptic fragment initially added (Table 1).

Inhibitory activity of reduced and alkylated peptic fragment, heat-treated pepsin fragment, lathyrilic rat skin collagen and CNBr-derived collagen fragments

Reduced and alkylated pepsin fragment still inhibited the reconstitution of C1 haemolytic activity almost to the same extent as the non-reduced fragment (Table 1). Both the untreated, and the reduced and alkylated, peptic fragment completely lost their inhibitory capacity when heated at 56°C for 30 min, then cooled at 4°C for 4 h before assay (Table 1). The reduced and alkylated pepsin fragment's inhibitory activity was more susceptible to heat-treatment than that of the untreated peptic fragment, as judged by heating at 46°C for 30 min, then cooling at 4°C, for

Table 1. *Effect of the peptic fragment, reduced and alkylated peptic fragment, heat-treated peptic fragment, collagen and collagen fragments on the reconstitution of C1 haemolytic activity*

Sample tested for inhibition of C1 haemolytic activity	Concentration of sample used (pmol/ml)	Inhibition (%)
Peptic fragment	60	90
Peptic fragment	2	42
Reduced and alkylated peptic fragment	60	80
Reduced and alkylated peptic fragment	2	32
Peptic fragment heated at 56°C for 30 min	120	0
Reduced and alkylated peptic fragment heated at 56°C for 30 min	120	0
Peptic fragment heated at 46°C for 30 min	60	44
Reduced and alkylated peptic fragment heated at 46°C for 30 min	60	8
Lathyratic rat skin collagen	67	0
CNBr-derived peptides of pig type II collagen	(2mg/ml)	0
Supernatant obtained after incubation of peptic fragment (60pmol/ml) with EAC4 cells	60	90
Supernatant obtained after incubation of peptic fragment (2pmol/ml) with EAC4 cells	2	39

24h, before assay (Table 1). Lathyratic rat skin collagen and CNBr-derived peptides of pig type II collagen showed no ability to cause inhibition of the reconstitution of C1 haemolytic activity at even very high concentrations relative to those that gave strong inhibition with the peptic fragment (Table 1).

Discussion

Chemical studies (Reid *et al.*, 1972; Reid, 1974, 1976) and electron-microscopy studies (Shelton *et al.*, 1972; Knobel *et al.*, 1975) have clearly shown that subcomponent C1q is composed of two very different types of protein structure, i.e. the six peripheral globular regions and the six collagen-like connecting strands plus fibril-like central portion. Physical, and further electron-microscopy, studies (Brodsky-Doyle *et al.*, 1976) have substantiated the proposal that 84% of the six collagen-like connecting strands plus fibril-like central portion of the molecule is composed of typical collagen-like triple-helical structure (Reid & Porter, 1976), and that this is the portion of the molecule resistant to peptic digestion at pH4.5 (Reid, 1976).

The binding of subcomponent C1q to immune aggregates appears to survive collagenase digestion, whereas the haemolytic activity of the subcomponent C1q is rapidly lost (Knobel *et al.*, 1974; Reid *et al.*, 1972). These results suggest that the six peripheral globular regions are involved in the binding of subcomponent C1q to immunoglobulin G. Pepsin digestion of subcomponent C1q at pH4.5 causes rapid proteolysis of the six globular regions, and the collagen-like pepsin fragment that is left is haemolytically inactive in assays involving the reconstitution of C1 activity. Moreover, under certain conditions, as shown in this present paper, the peptic fragment

inhibits the reconstitution of C1 haemolytic activity. Results presented in Table 1 indicate that the peptic fragment is unable to bind to erythrocytes coated with antibody. This inability of pepsin-digested subcomponent C1q to interact with immunoglobulin has been reported previously by Wiger & Natvig (1972). They studied the ability of subcomponent C1q to agglutinate latex particles coated with immunoglobulin G and found that there was complete loss of agglutination of the particles by subcomponent C1q after incubation of subcomponent C1q with pepsin for 2–3 h at 37°C at pH4.5.

Although the peptic fragment is haemolytically inactive, presumably owing to loss of the globular portions of the molecule that may be involved in binding to immunoglobulin, the results presented here strongly suggest that the collagen-like regions of subcomponent C1q can compete with intact subcomponent C1q in the utilization of a mixture of subcomponents C1r and C1s (Figs. 2 and 3). It has been shown that subcomponent C1s will bind to erythrocytes coated with antibody only in the presence of subcomponents C1q and C1r and Ca²⁺ (Ziccardi & Cooper, 1976b). Subcomponent C1r requires only subcomponent C1q and Ca²⁺ to bind to sensitized erythrocytes, or immune aggregates, and it readily binds to subcomponent C1s in free solution in the absence of subcomponent C1q (Ziccardi & Cooper, 1976b; Sim, 1976). It was found, in this present study, that at low concentrations of subcomponents C1q, C1r and C1s there was apparent interference (up to 80%) with the reconstitution of C1 haemolytic activity when the reconstitution was attempted in solution and the peptic fragment subsequently added, before the addition of EAC4 cells to monitor the amount of C1 formed. By using a 100–200-fold more concentrated solution of the subcomponents, efficient re-

constitution of haemolytic activity was obtained in solution that was not markedly affected (not greater than 10% inhibition) by subsequent addition of the peptic fragment. In contrast with what was found in the attempts to reconstitute component C1 in solution, at low concentrations of the subcomponents, no inhibition of C1 haemolytic activity reconstituted on EAC1q4 cells was observed if the peptic fragment was added after the subcomponent C1r-C1s mixture (Fig. 2). The same low concentrations and molar proportions of subcomponents C1q, C1r and C1s, and molar proportions of peptic fragment to subcomponents C1r and C1s, were used in both types of reconstitution experiment. It appears, therefore, that at these low concentrations of the subcomponents a stable complex, yielding haemolytically active C1, was readily formed on the cell surface but not in solution, which indicates that perhaps subcomponent C1q has a greater affinity for the subcomponent C1r-C1s complex when it is bound to antibody on the cell surface. Alternatively, there may be a requirement for interaction of a portion of the subcomponent C1r-C1s mixture with immunoglobulin, as well as with subcomponent C1q, to generate a stable component C1 complex on the cell surface.

The ability of the reduced and alkylated pepsin fragment to show strong inhibition of C1 haemolytic activity to almost the same extent as the non-reduced fragment (Table 1) was surprising, in view of the quite marked effects produced on the physical properties of the peptic fragment after reduction and alkylation, i.e. the lowering of the thermal transition temperature by 10°C relative to that of the non-reduced fragment and the inability of the reduced and alkylated fragment, unlike the non-reduced fragment, to regain collagen-like structure after heating to 56°C then cooling to 4°C (Brodsky-Doyle *et al.*, 1976). However, the inhibitory activity of the reduced and alkylated pepsin fragment was more sensitive to heating at 46°C than was the non-reduced fragment (Table 1), which is consistent with the 10°C difference in their thermal transition temperatures (Brodsky-Doyle *et al.*, 1976).

Solutions of lathyrin rat skin collagen, which contained single molecules of collagen in their native triple-helical structure, and the mixture of CNBr-derived peptides of pig type II collagen, which consisted of a mixture of peptides ranging from 33 residues to 366 residues long and which were probably glycosylated to the same extent as the collagen regions in subcomponent C1q, failed to inhibit the reconstitution of C1 haemolytic activity (Table 1). Therefore there does appear to be some specificity other than

simply a triple-helical structure, or a glycosylated repeating triplet sequence, required to bring about efficient inhibition of the reconstitution of C1 haemolytic activity.

Recent studies have indicated which roles the different structural portions of the subcomponent C1q molecule may play in platelet aggregation. Cazenave *et al.* (1976) have shown that subcomponent C1q inhibited platelet aggregation induced by collagen, and they postulated that subcomponent C1q competes with collagen for specific sites on the platelets. Wautier *et al.* (1976a) found that component C1 was attached to their platelet preparations and was involved in platelet aggregation by immune complexes or aggregated immunoglobulin. In further studies, involving the subcomponents of C1, Wautier *et al.* (1976b) have proposed that subcomponent C1q is attached to platelet-bound subcomponent C1s by its collagen-like portion, which leaves its non-collagen-like portion (the six peripheral globular regions) free to bind to immune complexes and thus cause platelet aggregation. Studies involving the peptic fragment of subcomponent C1q should help clarify the possible roles of subcomponent C1q in platelet aggregation, since this fragment might be expected to block the aggregation of platelets by collagen and also block the aggregation of EDTA-washed platelets by subcomponent C1q plus immune aggregates.

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