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The relationship between the binding ability and the rate of activation of the complement component C1

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Summary. The strength of the bond between C1 and C1 binders (as measured by C1q binding) has been correlated with the ability of the binders to activate C1. The rate of activation of C1 has been studied by following the extent of hydrolysis of the C1r and C1s subcomponents, using a purified preparation of C1 labelled with ^{125}I. The rate of activation of C1 was not correlated with the binding strength between C1q and the C1 binders. Immune complexes were found to activate C1 rapidly, whereas glutaraldehyde-aggregated IgG failed to activate faster than the spontaneous activation seen on incubation of C1 alone; the strength of the bond between C1q and the binders was similar in the two cases. It is suggested that an interaction other than the binding between C1q and C1 binders is necessary for activation of C1. C1 bound to immune complexes was not activated in the presence of $C\bar{1}$ inhibitor, indicating that the inhibitor can prevent the hydrolysis of C1r under the test conditions.

INTRODUCTION

The binding and activation of the complement component C1 is the initial stage in the activation of the classical complement pathway. The C1 molecule is a complex of three different proteins, C1q, C1r and C1s, possibly bound together by Ca^{2+} in the molar proportions of C1q/C1r/C1s = 1:4:4 (Gigli, Porter & Sim, 1976). Binding of C1 to immune complexes takes place through C1q, C1q is not altered following binding, but activation of the C1 molecule is accompanied by hydrolysis of the single polypeptide chains of C1r and C1s (both approx 83,000 mol. wt) to give two disulphide-linked chains of 56,000 ('heavy' chain) and 27,000 ('light' chain) mol. wt (Valet & Cooper, 1974; Sakai & Stroud, 1973; Ziccardi & Cooper, 1976), together with the appearance of proteinase catalytic sites on the 'light' chains in both cases. Dodds, Sim, Porter & Kerr (1978) have suggested that, following the binding of C1 to immune complexes through the C1q molecule, C1r is conformationally altered to the single chain intermediary C1r·, and this change exposes a catalytic site which hydrolyses other C1r molecules to the active $C\bar{1}r$ state, which in turn hydrolyses C1s to $C\bar{1}s$.

Borsos, Rapp & Walz (1964) showed that the binding of C1 to antibody-coated red cells was separable from the activation process, the former taking place rapidly even at 0°, whereas the subsequent activation was much slower and temperature-dependent, being negligible at 0° and continuing for several hours at 37°.

There have been several reports that the activation of C1 is not necessarily correlated with the extent of binding of C1. Allen & Isliker (1974) showed that immune complexes would no longer activate C1 after

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the tryptophane residues had been modified with 2-OH-5-NO_2-benzyl bromide, although binding of C1q was unimpaired. Füst, Bertók & Juhász-Nagy (1977) found that lipopolysaccharide endotoxin detoxified by gamma-irradiation would bind C1 but not activate it. In a later paper, Füst, Medgyesi, Rajnavölgyi, Csécsi-Nagy, Czikora & Gergely (1978) could find no correlation between the C1-binding ability of various immunoglobulin preparations treated in various ways and their capacity to activate C1. Curd & Cooper (1978) reported that polymers of Fc molecules would bind but not activate C1. Bartholomew & Esser (1978) showed that guinea-pig C1 binds directly to retroviruses in the absence of antibody but this binding does not result in activation, whereas when binding of C1 takes place through antibody specific for the virus, activation does take place.

The work presented here investigated the possibility that the rate and extent of activation of C1 by various C1 binders might be determined by the strength of the bond between C1q and the C1 binder. The extent to which C1 binders combine with C1 can be estimated by determining the functional affinity constant of the reaction between ^{125}I-labelled C1q and the C1 binder (Hughes-Jones & Gardner, 1978). The activation of C1 was estimated by following the rate of hydrolysis of ^{125}I-labelled C1r and C1s molecules, using a modification of the methods of Bartholomew & Esser (1977) and Cooper & Ziccardi (1977). The modification involves the labelling of purified C1 rather than the labelling of the C1s subcomponent with subsequent re-incorporation into the C1 molecule. No correlation was found between the values of the functional affinity constant and the degree of activation of C1.

MATERIALS AND METHODS

Preparation of C1

C1 was prepared from human serum by fractionation of a euglobulin precipitate on Sepharose 6B. The method used was based on that described by Gigli *et al.* (1976) with the exception that polyanethol sulphonate (10 μg/ml; Naff & Ratnoff, 1968) was substituted for diisopropyl phosphofluoridate as a C1 inhibitor. Column fractions were tested for C1 activity by measuring the haemolytic activity that could be restored to C1-depleted serum by addition of appropriate dilutions of the fractions, as described by Folkerd & Hughes-Jones (1979). The C1q content of the fractions containing C1 was determined using radial immunodiffusion with anti-C1q; purified C1q (Yonemasu & Stroud, 1971) was used as a standard.

C1 was labelled with ^{125}I by the lactoperoxidase technique (Heusser, Boesman, Nordin & Isliker, 1973). Aliquots of C1 (containing approx. 50 μg C1q) were labelled with 100 μCi ^{125}I. The unbound iodine was removed by dialysis at 0° against 10 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7·5, for 3 h with two changes of dialysis fluid. There was approximately a 30–50% uptake of the radioactivity by the C1; an aliquot containing 1 μg of C1q gave a counting rate of the order of 1×10^6 c.p.m. The labelled C1 was stored at −50° until required.

Preparation of C1 binders

IgG immune complexes. Complexes were obtained at equivalence using rabbit serum and a sheep serum with an IgG anti-rabbit IgG. The complexes were washed and suspended in a solution containing 10 mM Tris–HCl 150 mM NaCl and 5 mM $CaCl_2$, pH 7·5. The total IgG content was 5 mg/ml, as determined from the light absorption at 280 nm using the value of 14 for the $E^{1\%}_{1cm}$ of IgG after dissolving in 0·1 M NaOH.

Glutaraldehyde-treated red cells (glut-rbc). Human red cells were treated with glutaraldehyde as described by Hughes-Jones (1977). They were used as a 20% suspension.

Glutaraldehyde-treated IgG (glut-IgG). Human IgG (Lister Institute, Elstree, England) was aggregated by the action of glutaraldehyde (final concentration 0·1% g/100ml) using the technique of Avrameas & Ternynck (1969).

Antibody-coated sheep red cells (EA). Sheep cells were incubated with rabbit anti-sheep antibody as outlined by Lachmann & Hobart (1978), using an IgG rabbit anti-sheep serum (Burroughs Welcome).

Activation of ^{125}I-labelled C1

The extent of activation of ^{125}I-labelled C1 by C1 binders was investigated in the following way. ^{125}I-labelled C1 (approx. 1 μg C1q) in 20 μl of buffer containing 10 mM Tris–HCl, 5 mM $CaCl_2$, 150 mM NaCl, pH 7·5, was added to 180 μl of the same buffer containing the C1 binders under investigation. The mixture was incubated at 37° for various periods of time and the C1 binders then separated from the complex by centrifugation at 1700 ***g*** for 5 min. The C1 binders were then washed twice in ice-cold buffer of

the same composition as used for the reaction. Elution of the C1q, C1r and C1s molecules from the complexes was carried out at 0° by the addition of 200 μl of a buffer containing 20 mM EDTA, 50 mM Tris–HCl, 0·17 M NaCl (pH 7·0, conductivity 6·2 mS at 0°). An elution time of 5 min was found to be adequate. The supernatants were then separated from the binders by centrifugation and subjected to polyacrylamide gel analysis. In order to reduce non-specific absorption of labelled proteins, the glass tubes used for the experiments were filled with bovine serum albumin (5 mg/ml) for 16 h and then washed with saline before use.

Functional affinity constants and number of binding sites
The values of the functional affinity constants for the reaction between ^{125}I-labelled C1q and the various C1 binders together with the estimation of the total number of C1q binding sites was carried out by Scatchard analysis, as previously described (Hughes-Jones, 1977).

C1 Inhibitor (C1-In)
This was prepared by the method of Reboul, Arlaud, Sim & Colomb (1977) from human serum. The concentration of the C1 inhibitor was 360 μg/ml, as estimated by single radial immunodiffusion using an anti-C1 inhibitor (Behringwerke); normal serum was used as a standard, assuming a C1-In concentration of 240 μg/ml (Heimburger, 1975).

SDS-polyacrylamide gel electrophoresis
Gel analysis was carried out as described by Fairbanks, Steck & Wallach (1971) using either 7·5% or 10% gels; the 10% gels gave a better separation of the light chain of C1r from the A and C chains of C1q. Samples were reduced by incubation with an equal volume of 2% 2-mercaptoethanol, 8 M urea and 4% SDS in 0·2 M Tris–HCl, pH 8·0, at 65° for 10 min. Gels were stained with Coomassie blue. After staining, gels were sliced into 1·5 mm sections and the radioactivity measured. An alternative method of determining the distribution of radioactivity was by autoradiography of gel slabs, using intensification screens as described by Laskey & Mills (1977). A stacking gel (5%) was included in the gel slab technique. The relative amount of ^{125}I within each peak was determined by scanning the radiographs with a densitometer.

The molecular weights of the proteins were determined by reference to molecular weight markers run at the same time (polymers of lysozyme, Product No. 44223 2U; myoglobin and subunits, Product No. 44247; BDH, Poole, England).

RESULTS

Assessment of C1 activation

The ^{125}I-labelled C1 preparations were found to retain haemolytic activity when added to C1-depleted serum provided that the final H_2O_2 concentration during labelling was not greater than a 1:600,000 dilution of a 100 vol H_2O_2 solution. Above this concentration, haemolytic activity was rapidly lost.

The distribution of radioactivity after polyacrylamide gel analysis of reduced ^{125}I-labelled C1 is shown in Fig. 1. The identities of the ^{125}I-labelled polypeptides (gel *a*) were ascertained by comparison with the known molecular weights and labelling properties of C1 subcomponents (Ziccardi & Cooper, 1976; Cooper & Ziccardi, 1977; Sim, Porter, Reid & Gigli, 1977), and substantiated by the alteration in the peaks following activation of C1 by incubation at 37° for 16 h (gel *b*). Seven bands are visible by autoradiography. (1) A band at mol. wt 130,000, which is probably C1 inhibitor combined with the light chain of C1r. (2) A band at mol. wt 83,000, containing the uncleaved chains of C1r and C1s. (3) A band at mol. wt 80,000, which is an unidentified impurity; the amount present varied between different preparations. (4) A band at mol. wt 56,000, consisting of the combined heavy chains of activated C1r and C1s ($C\bar{1}r_H + C\bar{1}s_H$). (5) A band at mol. wt 36,000, which is the light chain of C1r ($C\bar{1}r_L$). The correct molecular weight of this chain is probably 26,000, but runs more slowly on electrophoresis (Sim *et al.*, 1977). (6) A minor band at mol. wt 30,000, which is the A chain of C1q. (7) The main band from C1q, represented by the C chain, at mol. wt 24,000.

An analysis of fourteen gels showed that on average 65% of the ^{125}I was distributed between the C1r and C1s molecules and the remaining 35% was combined with C1q. The light chain of C1r labelled to a greater extent than the other chains since the 36,000 mol. wt $C\bar{1}r_L$ peak usually contained either similar amounts or up to 50% more counts than the 56,000 mol. wt $C\bar{1}r_H + C\bar{1}s_H$ peak. Some activation of the C1 took place during purification and labelling, the activated molecules usually comprising some 10–20% of the total.

C1-binding activity

The ability of the C1 binders (immune complexes, glutaraldehyde-treated IgG, glutaraldehyde-treated

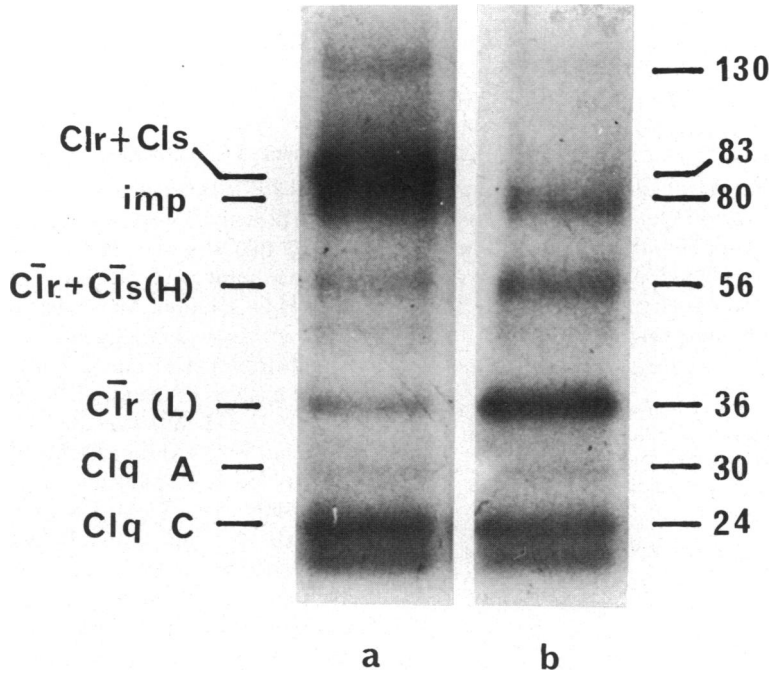

Figure 1. Autoradiograph of polyacrylamide gel electrophoresis of ^{125}I-labelled C1. Gel (a), immediately after thawing; gel (b), after 16 h incubation at 37°. C1r+C1s, inactivated intact chains of subcomponents; imp, unidentified impurity; $\overline{C1r}+\overline{C1s}$(H), heavy chains of activated subcomponents; C1r(L), light chain of activated $\overline{C1r}$; C1q A & C, respective chains of C1q. Figures give molecular weights $\times 10^{-3}$.

red cells and antibody-coated red cells) to bind C1 was measured by determining (1) the maximum amount of ^{125}I-labelled C1q that they were able to bind and (2) the value of the functional affinity constant (K) of the reaction. These measurements were carried out by standard Scatchard analysis and the results are given in Table 1. In the calculation of K, molar concentrations of C1q were used, but no account was taken of the probability that two or more globular heads were involved in the reaction. The values of the binding constants were similar to each other and fell within the range $0{\cdot}4$–$3{\cdot}4 \times 10^8$ M^{-1}. As found previously (Hughes-Jones & Gardner, 1978) the value for binding to glut-rbc was slightly higher than for the immune complexes.

Activation of C1

The ^{125}I-labelled C1q was activated by the addition of

Table 1. Functional affinity constants for C1q and C1 binders

C1 binder	Concentration*	Binding† capacity (μg/ml)	Final C1-binding‡ capacity (μg)	K (M^{-1})
Glut-IgG	10 mg/ml	520 (μg/ml)	51	$0{\cdot}5$–1×10^8 (av. $0{\cdot}8 \times 10^8$)
Glut-rbc	20% suspension	125	12	$1{\cdot}4$–$3{\cdot}4 \times 10^8$ (av.$2{\cdot}3 \times 10^8$)
Sheep rbc	20% suspension	5	$0{\cdot}5$	1×10^8 (1 experiment only)
Immune complexes	5 mg/ml	200	20	$0{\cdot}4$–$1{\cdot}5 \times 10^8$ (av.$0{\cdot}8 \times 10^8$)

* Concentration of the C1 binder preparation.

† Maximum binding capacity of 1 ml of the C1-binder for C1q as determined by Scatchard analysis.

‡ Total binding capacity of C1 binder for C1q in 200 μl of the reaction mixture used for activation. The amount of C1q in the ^{125}I-labelled C1 was approx. 1 μg in each experiment.

the C1 binders for various periods of time at 37°. The C1 binders were used in excess as given in Table 1, except in the case of EA. The extent of activation was estimated by EDTA elution of the ^{125}I-labelled C1 components from the C1 binders and subsequent determination by gel analysis of the extent of cleavage of C1r and C1s. The results have been analysed by determining the total number of counts in the following 3 peaks, (1) the uncleaved C1r+C1s peak (mol. wt 83,000), (2) the $C\bar{1}r_H + C\bar{1}s_H$ peak (mol. wt 56,000) and (3) the $C\bar{1}r_L$ peak (mol. wt 36,000) and calculating the distribution between the peaks, expressing the proportion in each peak as a percentage of the total number of counts in the three peaks. Figure 2 shows the extent of activation of the ^{125}I-labelled C1 with time, expressed as the proportion of counts remaining in the uncleaved C1r+C1s peak. It can be seen that there are very considerable differences between the rates of activation by the different C1 binders. The immune complexes brought about rapid activation, so that the proportion of counts in the cleaved C1r and C1s chains fell from 56% at 1 min to 20% of the total within 15 min, but thereafter the rate of activation was very much slower, 9% of the counts still being present in the C1r+C1s peak even after 20 h of incubation. On the other hand, the rate of activation of C1 by the glut-IgG aggregates was very slow and a similar rate of activation was seen in the controls when C1 was incubated alone. The glut-rbc brought about a gradual fall in the C1r+C1s peak, which still contained about 40% of the counts after 60 min. The sensitized sheep red cells brought about a more rapid rate of activation, although not as fast as the immune complexes over the initial 60 min period; after 5 h the sheep red cells and the immune complexes gave the same results, the C1r+C1s peak being reduced to 13%.

The fall in the radioactive content of the uncleaved C1r+C1s peaks was accompanied by a concomitant rise in the content of the $C\bar{1}r_H + C\bar{1}s_H$ and $C\bar{1}r_L$ peaks.

An assessment of the rate of activation can be obtained by drawing a tangent to the activation curves at time 0 and extrapolating to the base line. This gives an estimate of the average survival time of the uncleaved C1r and C1s molecules when bound to the C1 binders. Experimental error was too great to give an accurate estimate, but the approximate values were as follows: IgG complexes, 10 min; EA, 30 min; glut-rbc, 120 min; glut-IgG, 5 h.

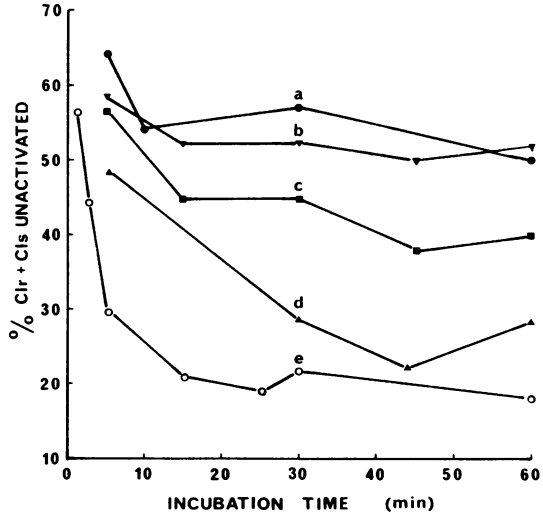

Figure 2. Extent of activation of C1 after incubation with four C1 binders, expressed as the amount of intact C1r and C1s chains remaining in the 83,000 mol. wt C1r and C1s peak. (a) Control in absence of C1 binder; (b), with glutaraldehyde-aggregated IgG; (c) with glutaraldehyde-treated red cells; (d), with sheep red cells sensitized with IgG anti-red cell antibody; (e), with immune complexes.

Effect of C1-inhibitor on C1 activation

The effect of $C\bar{1}$-In on the activation of C1 by both IgG complexes and glut-IgG was investigated by adding $C\bar{1}$-In in final concentrations between 1·2 and 120 μg/ml. After incubation, the complexes were washed with saline at 4° and then eluted with EDTA. Controls without inhibitor were similarly treated. It was necessary to incubate with glut-IgG for 16 h in order to obtain substantial activation. In the gel analysis of the eluates, the $C\bar{1}r_H + C\bar{1}s_H$ and $C\bar{1}r_L$ peaks could not be used, since the inhibitor reacts with the activated

Table 2. The effect of $C\bar{1}$-In on the extent of activation of ^{125}I-labelled C1 by both IgG complexes and glutaraldehyde-treated IgG

Activator	$C\bar{1}$-In (μg/ml)	Incubation time (h)	C1r+C1s/C1q ratio
IgG complexes	0	0·5	0·3/1
	1·2	0·5	4·9/1
	12	0·5	5·7/1
	60	0·5	3·8/1
Glut-IgG	0	16	1·4/1
	120	16	4·2/1

chains and dissociates them from the C1q (Arlaud, Sim, Duplaa & Colomb, 1979). The extent of activation was therefore determined from the amount of radioactivity left in the unactivated C1r+C1s peak, expressed as the ratio C1r+C1s/C1q to allow for variations in the amount of radioactivity applied to the gel. As can be seen from Table 2, in both experiments the amount of unactivated C1r+C1s eluted from the aggregates was considerably greater when inhibitor was present than when it was absent.

DISCUSSION

Considerable differences were found between the rates of activation of C1 by the four C1 binders that were investigated, but there was no correlation between the extent of activation and the values of the functional affinity constant for the reaction between C1q and the C1 binders. The most marked contrast was between the IgG–anti-IgG immune complexes on the one hand and the glut-IgG on the other, the former bringing about rapid activation during the first 15 min of incubation, whereas the latter brought about no detectable activation in excess of the spontaneous activation seen when C1 was incubated alone. The values obtained for the functional affinity constants were, nevertheless, similar for the two C1 binders, the average value being $0{\cdot}8 \times 10^8\ \text{M}^{-1}$ for both the IgG-immune complexes and for the glut-IgG. The highest binding constant found was that between C1q and glut-rbc and yet glut-rbc brought about a slower rate of activation compared to IgG complexes and to EA.

The failure of the binding constants to correlate with the rate of activation of C1 indicates that it is not only the binding of two of the globular heads of the C1q subcomponent of C1 to the C1 binder (Hughes-Jones & Gardner, 1978) which brings about the initiating conformational change of C1r to C1r' (Dodds *et al.*, 1978). An additional mechanism would appear to be involved, possibly involving an interaction with C1r or C1s with the C1 binder. There have been two reports that either C1r or C1s may be involved in the binding under certain circumstances. Goers, Ziccardi, Schumaker & Glovsky (1977) investigated a univalent antibody against polylysine and found evidence which suggested that the globular heads of C1q serve as anchors to bind C1 and that an additional site on the antigen–antibody complex binds to the central portion of C1q and to the C1r and C1s subcomponents and initiates activation. Esser, Bartholomew & Jensen (1979) found that, in the case of the binding of human C1 directly to retroviruses, activation only takes place when C1r is bound to the virion by C1q and by a second site located on C1s.

Goers *et al.* (1977) found that polylysine would activate a mixture of C1r and C1s and it is thus possible that a positive charge is involved in initiating autocatalysis. It is perhaps of significance that the positively-charged amino groups of lysine would have been blocked by glutaraldehyde in the glutaraldehyde-treated IgG; this might account for the failure of activation by this C1-binder.

When ^{125}I-labelled C1 was incubated with IgG aggregates in the presence of C1̄-inhibitor, it was found that considerably more of the unactivated C1r+C1s peak was present when compared to controls in the absence of inhibitor. This observation indicates the C1̄-inhibitor may prevent the autocatalysis of C1r, either inhibiting the formation of the single peptide intermediary, C1r' (Dodds *et al.*, 1978) or by reacting with C1r' and preventing the hydrolysis of C1r to C1r. The inhibition of hydrolysis of C1r and C1s by C1̄-inhibitor is consistent with the finding of Ziccardi & Cooper (1976) that C1-inhibitor would partially prevent the 'spontaneous' hydrolysis of a purified preparation of C1r. The concentration of C1 inhibitor found to prevent hydrolysis in the experiments described here was far lower than that found in the plasma, thus raising the question of how activation takes place in plasma in the presence of inhibitor. The control mechanism operating in plasma is probably more complex than that found in the isolated system.

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