Fixation of the First Component of Complement by Immune Complexes: Effect of Reduction and Fragmentation of Antibody

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The integrity of both covalent and non-covalent bonds between the heavy chains of rabbit immunoglobulin gamma antibody is essential for maximal efficiency of binding of the first component of complement to immune complexes.

Complement fixation by the classical pathway involves the binding of the first component of complement, C1, and it has been shown that this is effected by the binding of the subcomponent C1q to the C_H2* domain of immunoglobulins (Augener *et al.*, 1971; Connell & Porter, 1971; Colomb & Porter, 1975). Schur & Christian (1964) showed that antibody reduced with 10 mM-2-mercaptoethanol had only 40% of the efficiency to fix guinea-pig complement when complexed with antigen, compared with an efficiency of 100% for the unreduced antibody. However, the binding of antigen to its specific antibody was unimpaired by reduction.

I have investigated the correlation between the ability to bind the first component of complement and the number and location of the disulphide bonds broken in rabbit IgG anti-ovalbumin, since this could have some bearing on the hypothesis that complement binding by immune complexes necessitates a conformational change in the immunoglobulin molecule.

Materials and methods

Rabbit anti-ovalbumin IgG. Anti-ovalbumin serum was raised in rabbits as described by Porter (1955). IgG was isolated from pooled antisera (Wilkinson, 1969). The amount of antibody and the equivalence point were determined by a quantitative precipitin assay.

C₁-binding assay. The buffers used were saline-veronal-gelatin and dextrose-saline-veronal-gelatin, pH 7.4, as described by Nelson *et al.* (1966). Guinea-

pig C₁ was a euglobulin precipitate prepared from fresh guinea-pig serum by dialysis at 2°C for 2–3 h against water containing 5 mM-CaCl₂, adjusted to pH 7.4 with NaOH, until the ionic strength of the serum was 0.04. The precipitate formed was dissolved in saline-veronal-gelatin-5 mM-CaCl₂, pH 7.4, and stored at –70°C. C₁ was assayed by the haemolytic assay of Borsos & Rapp (1963) by using EAC4 cells prepared by the method of Borsos & Rapp (1967). One unit of C₁ if defined as the amount of C₁ needed to give an average of one haemolytic site/EAC4 cell in a suspension containing 1 × 10⁸ cells/ml. Immune complexes formed at equivalence were washed twice with 0.14 M-NaCl and suspended in 0.14 M-NaCl at 2°C. Guinea-pig C₁ was diluted to about 80 units/ml. An amount of immune complex sufficient to bind 50% of the C₁ was added to 1 ml of diluted C₁; the mixture was left at 2°C overnight and the complex removed by centrifugation. The amount of C₁ in the supernatant was determined.

Reduction and alkylation of antibody IgG. IgG (0.05 mM) in 0.1 M-Tris-HCl-2 mM-EDTA, pH 8.0, was reduced with dithiothreitol (0.1–5 mM) at room temperature for 1 h, followed by alkylation with iodoacetamide (2.5 × molar excess) at 2°C for 1 h. In some cases iodo[1-¹⁴C]acetamide was used.

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. Gels (5.6%, w/v) were used as described by Fairbanks *et al.* (1971). The partially reduced antibody was dissociated by heating at 80°C for 20 min in 4 M-urea-1% sodium dodecyl sulphate-0.1 M-Tris-HCl-0.1 M-iodoacetamide, pH 8, immediately before application to the gels. The gels were stained with Coomassie Blue (Weber & Osborn, 1969) and scanned with a Gilford Instruments gel scanner.

Isolation of polypeptide chains and fragments of IgG. Separation of the heavy and light chains of IgG was as described by Wilkinson (1969). Reaction with CNBr and separation of fragments was as described by Fruchter *et al.* (1970). Antibody Fab was prepared as described by Colomb & Porter (1975). Reduced Fab was prepared by reduction with

* Abbreviations: C_H1, C_H2 and C_H3, constant homology regions of the heavy chain (Fig. 1); IgG, immunoglobulin gamma; C₁, activated first component of complement; EAC4 cells, sheep erythrocytes coated with heated anti-(sheep erythrocyte) antibody together with the fourth component of complement; Fab, fragment antigen and complement binding; Dimer Fc, C-terminal half of the heavy chain with inter-heavy-chain disulphide bond intact; Fab, N-terminal half of heavy chain and light chain; pFc', C-terminal quarter of heavy-chain dimer; H, heavy chain; L, light chain; H_{Fab}, N-terminal three-quarters of the heavy chain.

Table 1. *Efficiency of binding of C_I to immune complexes*

The effect on the binding of C_I to immune complexes of partial reduction and fragmentation of antibody. The efficiency of binding is calculated from the relative amounts of complex required to bind the same amount of C_I compared with the binding by non-reduced antibody complex, which has an efficiency of 100%. The disulphide bonds reduced were calculated from radioactivity of the iodo[¹⁴C]acetamide-alkylated chains. The radioactivity on the heavy chains was corrected for the inter-heavy-light bonds reduced. n.d., Not done.

Molecular species of antibody in immune complex	Concn. of dithiothreitol used for reduction (mM)	Efficiency of C _I binding (%)	% of each molecular species deduced from polyacrylamide-gel electrophoresis			Disulphide bonds reduced/mol of IgG	
			IgG	HL	H and L	Heavy-chain bonds	Inter-light-heavy-chain bonds
IgG	0	100	100	0	0	0	0
IgG	0.1	81	66	32	2	0.35	0.06
IgG	0.5	43	15	58	27	1.6	0.6
IgG	1.0	22	0	26	73	2.45	1.7
IgG	5.0	17	0	0	100	n.d.	n.d.
Facb	0	60	Facb	H _{Facb} L	H _{Facb} and L	n.d.	n.d.
Facb	0.5	0.4	100	0	0	n.d.	n.d.
			0	24	76	n.d.	n.d.
Non-complexed fragments							
Facb	0	0.7					
Fc		0.6					

0.5mM-dithiothreitol, alkylation with iodoacetamide (2.5 × molar excess) and fractionation on a column of Sephadex G-150 in 10mM-Tris-HCl-0.14mM-NaCl, pH 7.4. Fc fragment was isolated as the disulphide-bond-linked dimer from a papain digest of IgG in the absence of cysteine (Dower *et al.*, 1975).

Results and discussion

Relationship between C_I binding by immune complexes and the number of antibody disulphide bonds reduced. Anti-ovalbumin was reduced with dithiothreitol (0.1–5mM) and alkylated, and the number of C_I units bound to immune complexes was determined. As a routine 0.1 µg of complex of non-reduced antibody was used as the control and the efficiency of binding by the reduced antibody complexes was calculated from the relative amounts of complex required to bind the same amount of C_I. The percentages of intact IgG molecules, molecules with inter-heavy-chain bonds reduced (heavy-light molecules) and free heavy and light chains were determined by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. In Table 1 the efficiency of C_I binding and the extent of reduction of the antibody is shown. The total number of disulphide bonds reduced on the heavy and light chains was determined by alkylating the partially reduced IgG with iodo[¹⁴C]acetamide and separating the chains after further reduction with 10mM-dithiothreitol and alkylation with iodoacetamide. The number of heavy chain and heavy-light disulphide bonds reduced,

calculated from radioactivity of the chains, is also shown in Table 1. A comparison of the results deduced from polyacrylamide-gel electrophoresis and radioactivity indicates that in addition to inter-heavy-chain bonds some intra-heavy-chain bonds are also reduced at concentrations of dithiothreitol of 0.5mM and higher.

Location of the disulphide bonds reduced in the heavy chain. It was assumed that the intrachain bond reduced was the labile one in the C_H1 domain (see Fig. 1) (O'Donnell *et al.*, 1970). To obtain direct evidence for this the CNBr fragments C-1, C-3 and C-4 (Fig. 1) were isolated from the radioactive heavy chains. All the radioactivity was found to be in the C-1 fragment. In a further experiment, antibody reduced with 0.5mM-dithiothreitol and alkylated with iodoacetamide was reduced with 10mM-dithiothreitol, alkylated with iodo[¹⁴C]acetamide, and the heavy and light chains were separated. When these heavy chains were treated with CNBr and the fragments C-1, C-3 and C-4 isolated, all the radioactivity was again found in the C-1 fragment. It can be concluded therefore that under the conditions of partial reduction, which results in loss in efficiency of C_I binding, the cystine residues of the C_H2 domain are not reduced nor do they undergo disulphide-bond interchange. Therefore the loss in C_I-binding efficiency is not due to a change in the disulphide-bond structure of the C_H2 domain, which is the domain that contains the binding site for C_I.

The efficiency of binding of C_I by immune com-

Fig. 1. Structure of rabbit IgG

Proteolytic fragments and polypeptide chains of rabbit IgG are indicated. The labile disulphide bonds are shown dashed. C_{H1}, C_{H2} and C_{H3} are the constant homology regions of the heavy chain and C-1, C-3, C-4 and C-5 are fragments produced by reaction of CNBr with heavy chain.

plexes decreases as the extent of reduction increases. The critical bond is probably the inter-heavy-chain bond since, under the mildest conditions of reduction when 34% of the molecules had their inter-heavy-chain bond reduced and other disulphide bonds essentially intact, the efficiency of binding of C_I by immune complexes was only 81% compared with that of intact molecules. However, the residual efficiency was in excess of the percentage of intact molecules, and when all the interchain bonds were reduced the immune complex was still able to bind C_I, although 6 times as much complex was required to bind the same number of units as were bound by the non-reduced antibody complex.

Non-covalent bonds between heavy chains. The heavy chains of IgG are held together by the inter-heavy-chain disulphide bond and also by non-covalent bonds within the Fc fragment. The pFc' fragment is a dimer at neutral pH, and therefore presumably there are non-covalent bonds between the heavy chains in this C-terminal homology region. The Fabc fragment lacks the C-terminal region (Fig. 1), and presumably the non-covalent binding between the heavy chains in Fabc will be less than in the intact molecule. Fabc in immune complexes binds C_I, but with an efficiency of 60% of that of the intact IgG. It was therefore decided to compare the efficiencies of C_I binding by antibody, Fabc and reduced Fabc fragment. However, Fabc reduced with 0.5mM-dithiothreitol, which reduced the inter-heavy-chain bond in 100% of the molecules (Table 1), did not precipitate with its specific antigen, and on a column of Sephadex G-150 (0.14M-NaCl, pH7.2) the elution volume was 1.4 times the elution volume of

the unreduced Fabc fragment. Presumably therefore reduced Fabc is a half-molecule at neutral pH and therefore a univalent antibody fragment.

Univalent antibody fragments, e.g. Fab fragments, have the same affinity for antigen as bivalent antibody molecules, but the complexes formed with antigen are soluble. The molar ratio of Fabc to ovalbumin at equivalence was 2:1. Reduced Fabc was therefore incubated with ovalbumin at the same ratio and also at a ratio of 4:1, and the ability to bind C_I of both these soluble complexes, and of reduced Fabc in the absence of antigen, was determined. The efficiency of C_I binding by the soluble immune complexes of reduced Fabc was only 1/200th that of the intact antibody complex, and reduced Fabc without antigen had a similar efficiency. This level of binding is in fact similar to that of dimer Fc or non-complexed Fabc alone (Table 1).

In conclusion, maximal binding of C_I to immune complexes of IgG antibody is dependent on the integrity of the inter-heavy-chain disulphide bond, and evidence is presented to show that the non-covalent binding between the two heavy chains in the C_{H3} region also plays a role in the efficiency of C_I binding by antibody in immune complex. However, the covalently linked dimer Fc fragment has a 200-fold lower binding efficiency compared with that of an immune complex. Therefore the interaction between antigen and antibody is the essential requirement for C_I binding, but the covalent and non-covalent bonds between the heavy chains are essential for maximal binding efficiency; this implies that the generation and/or stabilization of the binding site in the C_{H2} homology region is dependent on both factors.

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