USE OF AN IgG FRAGMENT PREPARED WITH PARTICULATE PLASMIN TO STUDY THE C1 BINDING AND ACTIVATION

G. J. ARLAUD, C. M. MEYER and M. G. COLOMB

DRF/Biochimie, Centre d'Etudes Nucléaires et Université Scientifique et Médicale, 85X, 38041 Grenoble-Cédex, France

Received 9 April 1976

1. Introduction

The fragment obtained by incubation of rabbit IgG with soluble plasmin, Facb, lacks the C-terminal C_H3 domain but retains the original ability of IgG to bind antigen and complement [1]. As contaminant traces of plasmin may contribute to the extrinsic activation of complement [2], we present here the results obtained with a Facb fragment prepared using solid phase-bound plasmin for the proteolysis of IgG. It appears that immune complexes formed with Facb prepared with particulate plasmin are able to bind and activate complement in a very similar way to complexes formed with IgG.

2. Materials and methods

IgG was purified from pooled rabbit serum as described previously [1] and subsequently treated with 2 mM DFP for 30 min at 30°C.

Abbreviations: Facb, fragment antigen and complement binding (this last property is shown when the fragment is involved in an immune complex); Fc, C-terminal half of the heavy-chain dimer; pFc', major C-terminal fragment released from IgG by pepsin or plasmin digestion; DFP, diisopropylphosphofluoridate; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BAEE, *N*-benzoyl-L-arginine ethyl ester; TAME, *p*-toluene sulfonyl-L-arginine methyl ester; SDS, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor.

The nomenclature of the complement components (C1, C1q, C1r, C1s) follows the W.H.O. recommendations. Enzymatic activities are expressed in nanokatals (nkat.) as recommended in the Enzyme Nomenclature (1973).

Rabbit immunisation, immune complexes between ovalbumin and IgG or Facb, SDS polyacrylamide gel electrophoresis, cyanogen bromide peptide preparation, C-terminal amino acid determination and tryptic peptide analysis were carried out as described previously [1].

$C1\bar{s}$ was purified according to Sakai and Stroud [3] with minor modifications. The 50% hemolysis time assay for complement was a modification of the technique described by Lachmann [4].

Double diffusion and single radial immunodiffusion in agarose were as described elsewhere [5].

The esterase activity of $C1\bar{s}$ was measured with 1.5 mM TAME, 0.9 mM BAEE and 1.0 mM ATEE in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl and 2 mM EDTA at 25°C as described previously [6,7].

2.1. *C1 binding assay*

Immune complexes were preformed in the presence of 2 mM DFP and kept suspended in 150 mM NaCl. Incubation with human serum was carried out at pH 7.5 in the presence of 2.5 mM $CaCl_2$ and 100 μg/ml SBTI at 0°C for various periods. All subsequent steps were performed at 0–4°C. After centrifugation at 3000 rev/min the resulting precipitate was washed twice with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$ and 50 to 100 μg/ml SBTI. The final precipitate was then suspended in the same medium and incubated at 30°C for 45 min to complete C1 activation. After centrifugation, the proteins bound to the immune complexes were extracted with 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10 mM EDTA and 50 μg/ml SBTI. The extracted proteins were analyzed by double diffusion, SDS polyacrylamide

North-Holland Publishing Company – Amsterdam

gel electrophoresis and measurement of the esterase activity.

2.2. *Preparation of plasmin*

Plasminogen was purified by affinity chromatography from pooled human citrated plasma according to a technique previously described [8] with slight modifications. After concentration by ultrafiltration and chromatography on Biogel P4, the yield was 40 to 50 mg protein starting from 500 ml of plasma. A further fractionation on a CM-cellulose column led to the separation of two allomeric forms of plasminogen as described previously [9]. Plasminogen was activated with urokinase (10 min at 25°C, enzyme to substrate ratio 1/200). The final yield of plasmin was 30 mg protein (specific activity: 33.3 nkat/mg protein, measured at 25°C with 1.0 mM BAEE as substrate in 50 mM Tris-HCl, pH 8.0). As both allomeric forms proteolyse IgG in the same way [10], the fraction eluted from Biogel P4 was used for all studies described here. Plasmin was coupled to Sepharose 4B essentially according to Axen et al. [11] with a protein to packed Sepharose ratio of 8 mg/ml.

2.3. *Preparation of Facb*

Rabbit IgG was brought to pH 2.5 with 1 M formic acid and incubated for 30 min at 25°C. The fraction was then poured onto a slurry of Sepharose-plasmin (plasmin to IgG ratio 1/2 to 1/5) and the pH raised to 8.0 with 2 M Tris-base. After incubation for 15 min at 25°C, the mixture was rapidly filtered under vacuum and the filtrate collected in the presence of SBTI.

For structural determinations, the incubation medium was purified on Sephadex G150 in 6 M urea, 0.2 M formic acid as described previously [1].

For complement binding assays, Facb was purified on Sephadex G150 in 15 mM sodium phosphate, 150 mM NaCl, pH 7.4.

All protein fractions were preserved by adding 0.1% sodium azide. All chemicals were analytical grade.

3. Results

3.1. *Chemical characterization of Facb*

The cleavage of IgG with plasmin led to a minimal yield of about 85% of Facb. A small amount of unsplit IgG was detected by SDS-polyacrylamide gel electrophoresis as shown in fig.1.

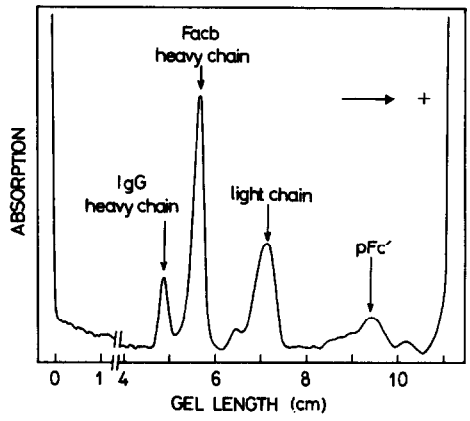

Fig.1. SDS-polyacrylamide gel electrophoresis of a plasmin digest of rabbit IgG. Reduced and alkylated sample (10 μg) was applied to the gel (5% acrylamide). The run laster 270 min. The gel was scanned after staining with Coomassie blue R250.

After cyanogen bromide treatment of Facb the C-terminal peptide C3 was purified and its structure compared with the corresponding peptide prepared from IgG in order to locate the split achieved by plasmin (fig.2). Following purification on Sephadex G150 in urea formic acid, the two C3 peptides were submitted to tryptic digestion as described previously [1]. High voltage electrophoresis at pH 1.9 and 3.6 enabled the discrimination of 14 tryptic peptides in C3 purified from IgG and 11 in C3 purified from Facb (fig.3).

The C-terminal analysis of C3 prepared from Facb gave with carboxypeptidase B a selective release of

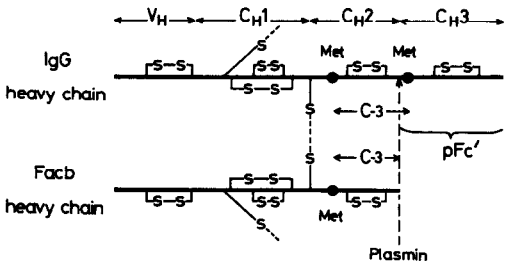

Fig.2. Compared structures of IgG and Facb heavy chains. The only methionine residues shown are those delimiting the CNBr peptide C3 used in the structural characterization of Facb. The disulfide bridge between heavy and light chains is only suggested.

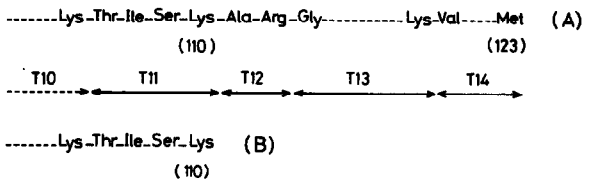

Fig.3. C-terminal part of C3 peptides. (A) IgG C3, (B) Facb C3. The residues are numbered from the inter-heavy-chain disulfide bond.

lysine whereas the analysis performed with a mixture of carboxypeptidase A and B led to a sequential release of lysine and serine corresponding to C-terminal amino acids of peptide T11 (fig.3). N-terminal determinations on pFc′ are not reported here, as plasmin can be responsible for secondary splits in pFc′ which are liable to lead to erroneous interpretations.

The main cleavage achieved by plasmin appears to be at a lysine residue located at 110 residues from the inter-heavy chain disulfide bond as was found previously for Facb prepared with soluble plasmin (fig.3).

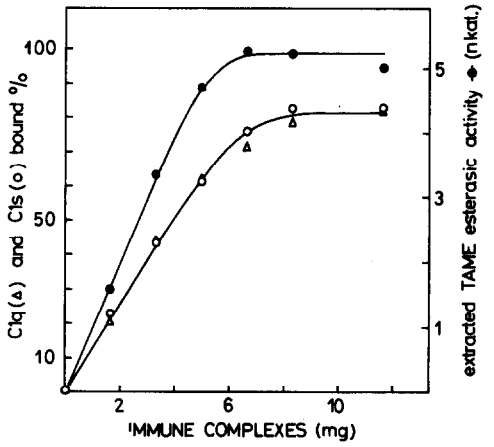

Fig.4. Fixation of C1q and C1s by IgG–ovalbumin complexes compared with extracted TAME esterase activity. Immune complexes (1.66 to 11.60 mg) were suspended in human serum (4.0 ml) and incubated for 45 min at 0°C. After centrifugation, C1q and C1s were assayed in the supernatants by radial immunodiffusion. The precipitates were treated as described in Materials and methods and the extracted $C1\bar{s}$ esterase activity was measured on TAME.

3.2. *C1 binding and activation by Facb-ovalbumin complexes*

The IgG and Facb fractions were applied to a Sephadex G150 column to eliminate major aggregates prior to complex formation. All measurements were carried out in parallel on IgG–ovalbumin, Facb–ovalbumin, and acid-treated IgG–ovalbumin complexes (in this last case, IgG was submitted to a preliminary incubation at pH 2.5 for 30 min at 25°C and then brought to pH 8.0, without addition of plasmin).

The esterase activity extracted was found to be a reliable estimate of the C1 bound as a complex, as illustrated by the good correlation between the amount of C1q and C1s bound and the $C1\bar{s}$ esterase activity extracted (fig.4). In the presence of 10 mM EDTA, C1 was disrupted and C1q alone was bound to immune complexes.

Table 1
Comparison of the esterase activity extracted from Facb–ovalbumin, IgG–ovalbumin and acid-treated IgG–ovalbumin. Identity with $C1\bar{s}$

	Extracts			Purified
	IgG	Acid-treated IgG	Facb	$C1\bar{s}$
TAME/BAEE	3.83	3.68	3.73	3.86
ATEE/TAME	0.73	0.72	0.72	0.73
BAEE/ATEE	0.36	0.38	0.37	0.35

Immune complexes (8.0 mg) were suspended in human serum (4.0 ml) for 45 min at 0°C. Each result represents the ratio of esterase activity towards two different substrates. Other conditions as described in Materials and methods.

The esterase activity eluted from the three different ovalbumin complexes was unambiguously identified as $C1\bar{s}$ (table 1). With three different substrates this activity appears to behave exactly as purified $C1\bar{s}$. Under the conditions used. $C1\bar{r}$ esterase activity did not seem to be detected.

The incubation of C1 still bound to the immune complexes for 45 min at 30°C led to the recovery of fully activated $C1\bar{s}$ after extraction and disruption of $C\bar{1}$. When this final activation step was omitted, the activation level of extracted $C1\bar{s}$ showed large variations between 30% and 80%.

The analysis of the extracts by double diffusion as well as their SDS-polyacrylamide gel electrophoresis pattern indicated the almost exclusive presence of C1q, $C1\bar{r}$ and $C1\bar{s}$. The extraction yield of the C1 components, based on $C1\bar{s}$ was approx. 35 to 40%.

In the case of Facb–ovalbumin complexes, an additional binding of plasmin and/or plasminogen was detected by double diffusion. The presence of SBTI throughout the binding and elution steps prevented any interference of plasmin in the esterase assay.

The binding of C1 as a function of the amount of immune complexes followed a very similar evolution for the three kinds of complexes. Facb–ovalbumin complexes appeared to bind 70 to 75% of the C1 bound by IgG–ovalbumin complexes (fig.5).

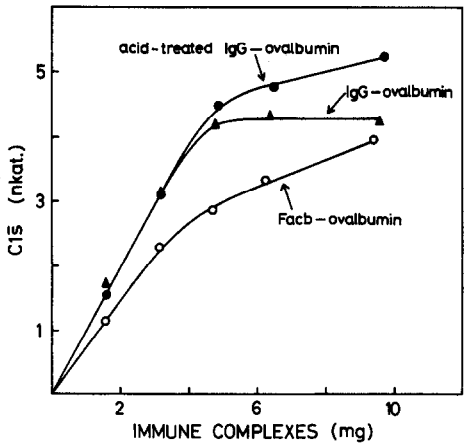

Fig.5. Comparison of the $C1\bar{s}$ esterase activity extracted from Facb–Ovalbumin and reference immune complexes. Immune complexes (1.56 to 9.72 mg) were suspended in human serum (4.0 ml) and treated as described in Materials and methods. $C1\bar{s}$ esterase activity was measured on TAME.

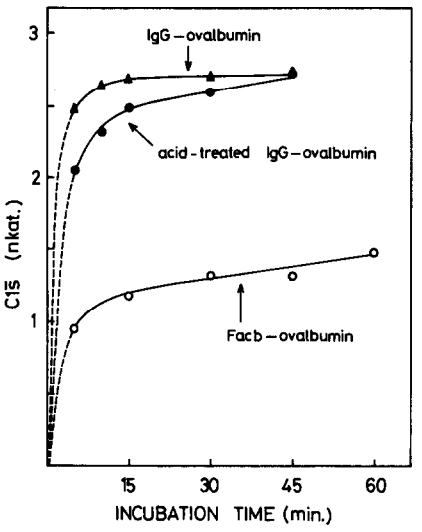

Fig.6. Fixation of human C1 by Facb–ovalbumin and reference immune complexes as a function of the incubation time at 0°C. Immune complexes (Facb–ovalbumin: 3.13 mg; IgG–ovalbumin: 3.20 mg acid-treated IgG–ovalbumin: 3.24 mg) were suspended in human serum (4.0 ml), incubated for 5 to 60 min at 0°C and centrifuged. The precipitates were treated as described in Materials and methods. TAME was used to measure $C1\bar{s}$ esterase activity.

The kinetics of binding was also very similar for the different complexes (fig.6) with a rapid phase (about 10 min) followed by a much slower binding. This seems to indicate a high affinity of C1 for the immune complexes.

These results were confirmed by the hemolytic assay reflecting the level of C1 remaining in the serum after fixation by the different immune complexes, IgG–ovalbumin, acid-treated IgG–ovalbumin, and Facb–ovalbumin gave respectively a relative binding of 100%, 77% and 63%.

The activation of C1 by Facb–ovalbumin complexes appeared to be promoted in the same way as by the two other ovalbumin complexes. This observation was based on the rapid kinetics of activation of C1 bound to Facb–ovalbumin complexes upon incubation to reach maximal activation of C1.

4. Discussion

These results show that the Facb fragment of IgG

can contribute to the study of the binding and activation of C1. They rule out an absolute prerequisite for the integrity of the entire Fc for the activation of C1 as was postulated previously [12]. These results confirm other preceeding observations on Facb prepared with soluble plasmin [1,13].

The differences in binding properties observed between Facb–ovalbumin complexes and reference complexes seem however to show that the C_H3 domain of IgG may also contribute to the binding of C1. This domain may interact with C1r as an interaction between C1r and an IgG-Sepharose system was reported previously [14]. This would strengthen the major interaction between C1q and the C_H2 domain [15].

The affinity of plasmin for the Facb–ovalbumin complexes underlines the absolute requirement for particulate plasmin to prepare Facb and for the presence of selective inhibitors of plasmin in the media used for the study of C1 activation.

Acknowledgements

This investigation was supported by research grants from the Centre National de la Recherche Scientifique (ERA No. 36), the Délégation Générale à la Recherche Scientifique et Technique (Contrat No. 75.7.0747) and the Fondation pour la Recherche Médicale.

References

[1] Colomb, M. G. and Porter, R. R. (1975) Biochem. J. 145, 177–183.
[2] Cooper, N. (1973) in: Contemporary Topics in Molecular Immunology (Reisfeld, R. A., ed.) Vol. 2, pp. 155–183, Plenum, New York.
[3] Sakai, K. and Stroud, R. M. (1973) J. Immun. 110, 1010–1020.
[4] Lachmann, P. J., Hobart, M. J. and Aston, W. P. (1973) in: Handbook of Experimental Immunology (Weir, D. M., ed.) Vol. 1, pp. 5–10, Blackwell, Oxford.
[5] Ouchterlony, Ö. and Nilsson, L. Å. (1973) in: Handbook of Experimental Immunology (Weir, D. M., ed.) Vol. 1, pp. 19.1–19.39, Blackwell, Oxford.
[6] Hazen, G. G. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) Vol. 2, pp. 1000–1057, Academic Press, New York.
[7] Valet, G. and Cooper, N. R. (1974) J. Immun. 112, 339–350.
[8] Brockway, W. J. and Castellino, F. J. (1972) Arch. Biochem. Biophys. 151, 194–199.
[9] McClintock, D. K., Englert, M. E., Dziobkowski, C., Snedeker, E. H. and Bell, P. H. (1974) Biochemistry 13, 5334–5344.
[10] Arlaud, G., Meyer, C. and Colomb, M. G. (1975) 10th FEBS Meeting, Paris, Abstract No. 1590.
[11] Axén, R., Porath, J. and Ernback, S. (1967) Nature 214, 1302–1304.
[12] Allan, R. and Isliker, H. (1974) Immunochemistry 11, 243–248.
[13] McLennan, I. C. M., Connell, G. E. and Gotch, F. M. (1974) Immunology 26, 303–310.
[14] Assimeh, S. N. and Painter, R. H. (1975) J. Immun. 115, 488–494.
[15] Kehoe, J. M. and Fougereau, M. (1969) Nature 224, 1212–1213.