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Subfragmentation of the Fc fragment of human IgG1 myeloma protein by thermolysin

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Summary. Human IgG1 Fc fragment was digested at neutral pH by thermolysin, producing two large subfragments: one comprising the major part of the Fc fragment but devoid of the hinge region; the other comprising the Cγ3 domain. The former fragment retained the capacity to react with 'general' rheumatoid factors whereas the latter did not, indicating that the binding site for 'general' rheumatoid factors on the Fc fragment of human IgG1 does not involve the hinge region of the molecule.

INTRODUCTION

Enzymatic cleavage studies have proved useful in the location of the binding site of rheumatoid factors to the IgG molecule (Goodman, 1961; McDuffie, Oikawa and Nishi, 1965). In this manner, the Fc fragment of human IgG has been shown to contain the antigenic determinant against which 'general' (non-anti-allotypic) rheumatoid factors are directed (Stewart, Hunneyball and Stanworth, 1975); whereas the smaller pFc′ fragment (produced by pepsin digestion of IgG) which comprises the C-terminal half of the Fc fragment (Cγ3 domain) has been found to lack such activity. It would appear, therefore, that the binding site for these antiglobulins may be located within the N-terminal half of the Fc region (Cγ2 domain). Further subfragments of the Fc fragment are now required to confirm this inference. In a previous paper (Hunneyball and Stanworth, 1975) we investigated the properties of a new lysine-specific protease with regard to cleavage of human IgG in an attempt to produce subfragments of the Fc region. This approach has been extended using the enzyme thermolysin (derived from *Bacillus thermoproteolyticus* Rokko) which hydrolyses peptide bonds N-terminal to hydrophobic residues with bulky side chains, with a relatively high degree of specificity (Matsubara, Sasaki, Singer and Jukes, 1966; Matsubara and Sasaki, 1968). Digestion of the IgG1 Fc fragment with thermolysin produced two major subfragments: one corresponding to the major part of the Fc fragment but devoid of the hinge region, the other corresponding to the Cγ3 domain of the molecule. Studies with sera from patients with rheumatoid arthritis indicated that the site of binding of 'general' rheumatoid factors on the Fc fragment is not located in the immediate vicinity of the hinge region of the molecule.

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MATERIALS AND METHODS

Preparation of human IgG and Fc fragments

Pooled human IgG was isolated from serum by precipitation with 33 per cent saturated ammonium

sulphate and then purified by batch ion-exchange chromatography using diethylaminoethyl-cellulose (Whatman DE 52) (Stelos, 1967; Stanworth, 1960). IgG1 myeloma proteins were prepared in an identical manner. Purity of the IgG preparations was assessed by immunoelectrophoresis and analytical ultracentrifugation at a concentration of 10 mg/ml. In addition, the IgG1 myeloma proteins appeared pure by both immunodiffusion using subclass-specific antisera and Gm typing. The Fc fragments of IgG1 and pooled IgG were prepared by papain digestion in the presence of cysteine. IgG (10 mg/ml) in 0·075 M phosphate buffer, pH 7·0, containing 0·075 M NaCl, 0·002 M disodium EDTA and 0·01 M cysteine, was incubated for 1·5 h in the presence of papain (Sigma Chemical Company) at an enzyme substrate ratio of 1:100 by weight. Digestion was terminated by addition of *N*-ethyl maleimide to a final concentration of 0·01 M and the digestion products separated by gel filtration on a column (90 × 3·2 cm) of Sephadex G-150 equilibrated with 0·05 M ammonium carbonate buffer, pH 8·6. The Fc fragment was freed from contaminating Fab fragment by ion-exchange chromatography on carboxymethyl Sephadex (Sephadex C50, Pharmacia, Ltd) and diethylaminoethyl cellulose (Whatman DE 52) utilizing stepwise elution, according to the method of Franklin and Prelli (1960). The Fc preparation was found to be pure by both immunoelectrophoresis and analytical ultracentrifugation at a concentration of 10 mg/ml.

Antisera

Sheep antisera against the human IgG Fc fragment were kindly supplied by Dr D. Catty (Department of Experimental Pathology, University of Birmingham) and purified by absorption with pure Fab fragment. Specific anti-Cγ2 domain antiserum was prepared by absorbing sheep anti-Fc antiserum with pFc′ fragment (Cγ3 domain), which had been prepared according to the method of Turner and Bennich (1968) and insolubilized using ethylchloroformate according to Avrameas and Ternynck (1967). Antiserum to the Cγ3 domain was prepared by injecting guinea-pigs with pFc′ fragment in Freund's complete adjuvant. Baboon antiserum to sheep erythrocytes was kindly produced by Mr G. A. Stewart (Department of Experimental Pathology, University of Birmingham).

Rheumatoid arthritis sera were obtained from patients with typical seropositive rheumatoid arthritis, from the Queen Elizabeth Hospital (Birmingham) by courtesy of Dr C. F. Hawkins.

Miscellaneous reagents

Thermolysin (EC 3.424.4) was purchased from Serva Fine Biochemicals Company as a thrice crystallized preparation stabilized with 40 per cent sodium acetate and calcium acetate. Sheep erythrocytes were obtained from Burroughs Wellcome Ltd, as sheep blood in Alsever's solution. Unless otherwise stated, all general laboratory reagents were obtained as 'Analar' grade from B.D.H., Ltd.

Immunoelectrophoresis and double diffusion

Immunoelectrophoresis was performed with a Shandon electrophoresis apparatus using 1 per cent Oxoid Agar No. 3 in barbitone buffer, pH 8·6. (I = 0·05). Electrophoresis was carried out at a constant current of 18 mA per plate for 1·5 h. Immunodiffusion was allowed to proceed for 24 h at 4°.

Amino acid analysis

The total amino acid content of protein fractions was determined by the method of Spackmann, Stein and Moore (1958), using a Jeolco JLC 5AH automatic amino acid analyser. Protein hydrolyses were performed for 24, 48 and 72 h, corrections being made for destruction during hydrolysis of amino acids such as serine and threonine and the incomplete release of amino acids such as isoleucine and valine during shorter hydrolysis times.

N-terminal analysis was performed according to the method of Gray (1972) employing dansyl chloride.

Molecular weight determinations

Molecular weights were determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis at pH 7·0 in the presence of 2-mercaptoethanol by the method of Weber and Osborn (1969), employing 10 per cent acrylamide gels and staining with Coomassie brilliant blue R (Gurr Ltd).

Reactivity with 'general' rheumatoid factors

The reactivity of human IgG subfragments with 'general' rheumatoid factors was measured by a haemagglutination–inhibition technique employing diluted rheumatoid arthritis serum and sheep erythrocytes sensitized with a subagglutinating dose of baboon anti-sheep erythrocyte antiserum as

described previously by Stewart *et al.* (1975). Prior to testing, the rheumatoid sera were clarified by centrifugation, decomplemented by incubation at 56° for 20 min and absorbed with an equal volume of packed sheep erythrocytes. The rheumatoid serum was diluted with 0·01 M phosphate-buffered saline (0·15 M), pH 7·2, to give an agglutination titre of 1 in 4 with sensitized erythrocytes. To serial doubling dilutions (0·025 ml) of inhibitor (at an initial concentration of 10 mg/ml) in the wells of a microtitre tray was added an equal volume of the diluted rheumatoid serum. After incubation for 1 h at 37°, an aliquot (0·025 ml) of a 1 per cent suspension of sensitized cells was added to each well. The inhibition titres were read after incubation for a further hour.

Gm typing

The expression of allotypic markers on myeloma proteins and subfragments was determined by a haemagglutination–inhibition technique employing human O Rh^+ erythrocytes sensitized with incomplete anti-D antibodies of known Gm type and sera from normal transfused blood donors.

RESULTS

Thermolysin digestion conditions

The IgG1 Fc fragment from myeloma protein Cau was digested with thermolysin by a method similar to that described by Matsubara (1970). Fc fragment (20 mg/ml) in 0·01 M Tris–HCl buffer, pH 8·0, containing 0·002 M calcium acetate, was digested with thermolysin at 37° at an enzyme: substrate ratio of 1:50 by weight. Serial samples were removed between 10 sec and 24 h and the thermolysin inactivated by addition of an equal volume of 0·01 M disodium EDTA in 0·01 M phosphate buffer, pH 7·0. The samples were examined by immunoelectrophoresis at pH 8·6, from which the optimum digestion time was estimated to be 3 h. This time was used for subsequent digestions. The digestion products were separated by gel filtration on a column (120 × 2·4 cm) of Sephadex G-100 equilibrated with 1·0 M acetic acid containing 0·025 M NaCl. Four main fractions could be distinguished from the elution profile (Fig. 1a). Fractions 1, 2 and 3 were collected, neutralized, dialysed against 0·01 M phosphate-buffered saline (0·15 M) pH 7·2, and concentrated by ultrafiltration. The Sephadex G-100 elution profile indicated that fraction 4, which contained dialysable material, was heterogeneous containing at least two components. Fraction 4 was therefore rotary evaporated (at 40°) to a small volume and separated on a column (90 × 2·2 cm) of Sephadex G-10 equilibrated with 0·01 M ammonium hydroxide. Three fractions could be distinguished from the elution profile: 4A, 4B and 4C (Fig. 1b). These were collected, rotary evaporated and dissolved in a small volume of 0·01 M phosphate-buffered saline (0·15 M).

Samples of Fc fragments prepared from IgG1

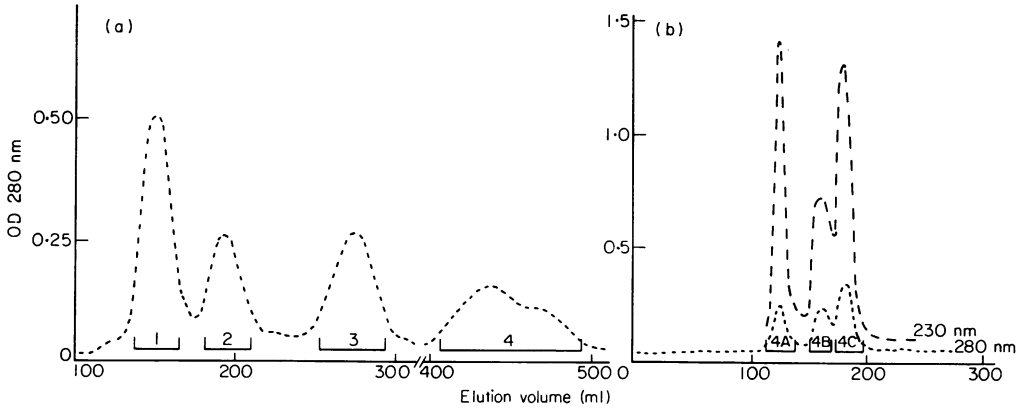

Figure 1. (a) Gel filtration of a 3-h thermolysin digest of human IgG1 Fc fragment on a column (120 × 2·4 cm) of Sephadex G-100 equilibrated with 1·0 M acetic acid contaning 0·025 M sodium chloride. (b) Gel filtration of Sephadex G-100 fraction 4 of a 3-h thermolysin digest of human IgG1 Fc fragment on a column (90 × 2·2 cm) of Sephadex G-10 equilibrated with 0·01 M ammonium hydroxide.

myeloma proteins of Gm 'non-a' type and normal pooled IgG, digested under identical conditions to those used for the IgG1 Gm(a) protein (Cau) described previously, gave elution profiles on Sephadex G-100 identical to that exhibited by the digest of the IgG1 Gm(a) myeloma protein (Cau) which was used for subsequent studies on the digestion products.

Characterization of digestion products

Immunodiffusion

The four fractions from Sephadex G-100 gel filtration were initially characterized by gel diffusion against specific antisera (Table 1) and immunoelectrophoresis using anti-Fc antiserum. Fractions 1 and 2 showed reactions of complete identity with the Fc fragment on gel diffusion against anti-Fc, anti-Cγ2 domain and anti-Cγ3 domain antisera (Fig. 2a, b). Fraction 3 showed a reaction of partial identity with fractions 1 and 2 and the Fc fragment on gel diffusion against anti-Fc, indicating an antigenic deficiency in comparison to the Fc fragment. The lack of precipitation between fraction 3 and anti-Cγ2 domain antiserum indicates that this fraction contains only the Cγ3 domain, whereas fractions 1 and 2 contain the major part, if not all, of the Cγ2 domain in addition to the Cγ3 domain. Neither fraction 4 nor the purified subfractions of fraction 4 (4A, 4B and 4C) precipitated with any of the antisera, a phenomenon consistent with these fractions containing small peptides. Immunoelectrophoresis of fractions 1, 2 and 3 (Fig. 2c) indicated that both fraction 1 and fraction 2 possessed identical electrophoretic mobility to the original Fc fragment. Fraction 3, however, was more anodic, its electrophoretic arc being positioned symmetrically about the well, indicating an electrophoretic mobility similar to those of the pFc′ and tFc′ fragments reported by Matthews, Stewart and Stanworth (1971). In order to confirm the apparent similarity between fraction 3 and the pFc′ and tFc′ fragments (as characterized by Matthews *et al.*, 1971), gel diffusion analysis was performed with fraction 3, pFc′ and tFc′ fragments against anti-Fc and anti-Cγ3 domain antisera (Fig. 2d). Fraction 3 gave a reaction of complete identity with both the pFc′ and tFc′ fragments against both antisera.

Table 1. Gel diffusion precipitin analysis of the thermolysin digestion products of IgG1 Fc fragment (at a protein concentration of 5 mg/ml) using specific antisera

Antiserum	Fr. 1	Fr. 2	Fr. 3	Fr. 4
Sheep anti-Fc	+	+	+	−
Sheep anti-Cγ 2 domain	+	+	−	−
Guinea-pig anti-Cγ 3 domain	+	+	+	−

Table 2. Sedimentation coefficients of thermolysin subfragments of IgG1 Fc fragment at a concentration of 3 mg/ml in both neutral buffer (0·01 M phosphate-buffered saline, 0·15 M, pH 7·2) and an acidic buffer (1 M acetic acid containing 0·025 M sodium chloride)

Sample	$S_{20,w}$	
	Neutral buffer	Acidic buffer
Fr. 1	4·18±0·05	2·60±0·03
Fr. 2	3·93±0·03	1·98±0·03
Fr. 3	2·29±0·03	1·56±0·03

Analytic ultracalentrifugation

Fractions 1, 2 and 3 were analysed in the ultracentrifuge (M.S.E. Centriscan) at a concentration of 3 mg/ml in both a neutral buffer (0·01 M phosphate-buffered saline, 0·15 M, pH 7·2) and an acidic buffer (1·0 M acetic acid containing 0·025 M NaCl), the results of which are summarized in Table 2. In the neutral buffer, fractions 1 and 2 sedimented at a similar rate to the undigested Fc fragment, whereas fraction 3 sedimented at a similar rate to that reported for the pFc′ fragment (Heimer, Schnoll

Table 3. Molecular weight determinations of thermolysin subfragments of human IgG1 Fc fragment determined by SDS polyacrylamide gel electrophoresis in the presence and absence of reducing agent (2-mercaptoethanol)

Sample	Molecular weight		
	In the absence of 2-mercaptoethanol	In the presence of 2-mercaptoethanol	Combined molecular weight of dimeric fragment
Fc	56,000±1000	27,000±1000	54,000±2000
Fr. 1	57,000±1000	27,500±1000	55,000±2000
Fr. 2	27,500±1000	27,000±1000	54,000±2000
Fr. 3	11,500±500	11,500±500	23,000±1000

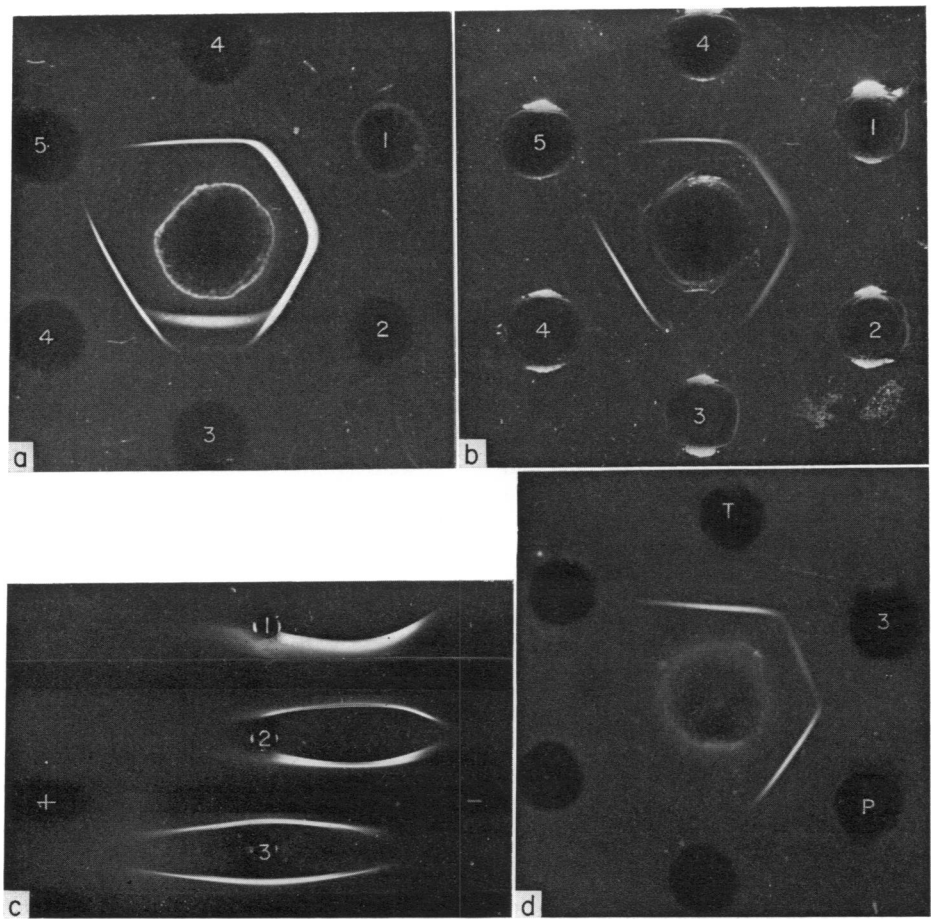

Figure 2. (a) Gel diffusion analysis of Sephadex G-100 fractions 1, 2 and 3 of a 3-h thermolysin digest of human IgG1 Fc fragment against sheep anti-Fc antiserum. Sample 4, Fc fragment; sample 5, buffer. (b) Gel diffusion analysis of Sephadex G-100 fractions 1, 2 and 3 of a 3-h thermolysin digest of human IgG1 Fc fragment against sheep anti-Cγ2 domain antiserum. Sample 4, Fc fragment; sample 5, buffer. (c) Immunoelectrophoresis of Sephadex G-100 fractions 1, 2 and 3 of a 3-h thermolysin digest of human IgG1 Fc fragment, using sheep anti-IgG antiserum. (d) Gel diffusion analysis of Sephadex G-100 fraction 3 of a 3-h thermolysin digest of human IgG1 Fc fragment together with pFc′ (P) and tFc′ (T) fragments of pooled human IgG against guinea-pig anti-Cγ3 domain antiserum.

and Primack, 1967; Turner and Bennich, 1968; Matthews *et al.*, 1971). Fraction 2 sedimented at a slightly slower rate than fraction 1, which is consistent with it being smaller than fraction 1. In the acidic buffer, all three fractions gave lower sedimentation coefficients due to electrostatic repulsion between the two polypeptide chains at the low pH. However, in this dissociating buffer, the sedimentation rate of fraction 2 was markedly lower than that of fraction 1. This finding is consistent with the difference in the position of elution from Sephadex G-100 in 1·0 M acetic acid shown by fractions 1 and 2, indicating that the latter probably constitutes a non-covalently linked dimer, whereas fraction 1 would appear to be covalently linked like the undigested Fc fragment. In order to confirm these conclusions, SDS polyacrylamide gel electrophoresis was performed on the fragments in the presence and absence of reducing agent (2-mercaptoethanol) using the method of Weber and Osborn (1969). The results obtained (given in Table 3) substantiated the ultracentrifuge data, indicating

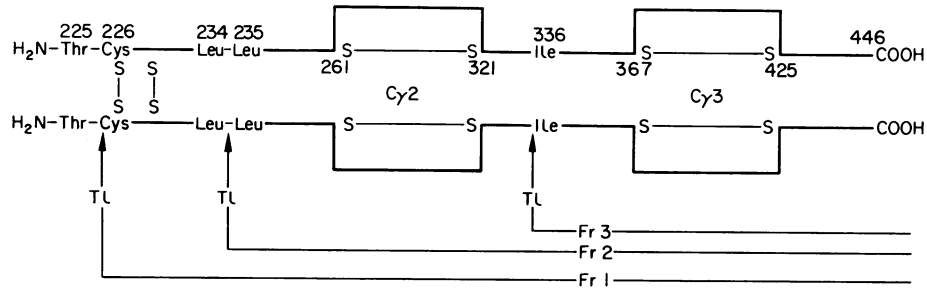

Figure 3. Diagrammatic representation of the Fc fragment of human IgG1 showing the probable positions of cleavage by thermolysin. Sequence numbering based on Edelman, Cunningham, Gall, Gottlieb, Rutishauser and Waxdal (1969).

that fraction 1 was identical in size to the undigested Fc fragment, whereas fraction 2 appeared to be of similar size, but non-covalently bonded, and thus dissociated to single chains in SDS or acetic acid. Fraction 3 also appeared to be non-covalently bonded, each individual polypeptide chain comprising half of the individual Fc chains. This is consistent with fraction 3 comprising the Cγ3 domain.

Table 4. Amino acid composition of fractions 1 and 2 obtained by thermolysin digestion of the human IgG1 Fc fragment

Amino acid	Fraction 1		Fraction 2		EuFc†
	Residues/ mole	NEI*	Residues/ mole	NEI*	
Ala	16·7	16	14·3	14	14
Arg	9·7	10	8·1	8	12
Asp	41·6	42	42·2	42	42
Cys	12·3‡	12	8·1‡	8	12
Glu	52·6	52	52·8	52	50
Gly	23·8	24	23·6	24	20
His	8·4	8	8·7	8	12
Ile	7·5	8	8·1	8	8
Leu	34·0	34	32·1	32	34
Lys	35·8	36	35·7	36	36
Met	8·0	8	7·4	8	6
Phe	13·8	14	14·2	14	14
Pro	44·3	44	40·6	40	44
Ser	40·5	40	40·3	40	40
Thr	28·3	28	27·5	28	28
Trp	n.d.	n.d.	n.d.	n.d.	8
Tyr	18·8	18	18·6	18	18
Val	46·0	46	45·8	46	46

n.d. = Not determined.

* Nearest even integer.

† Based on Edelman *et al.* (1969) residues 225–446.

‡ Determined as cysteic acid after performic acid oxidation.

Amino acid analysis

N-terminal analysis indicated cysteine as the N-terminal amino acid of fraction 1, in contrast to N-terminal threonine in the undigested Fc fragment. Fractions 2 and 3 appeared to possess leucine and isoleucine respectively at their N-termini. The amino acid content of fractions 1 and 2 is shown in Table 4. The amino acid composition of fraction 2 was similar to that of fraction 1, but deficient in fourteen residues (four proline, four cysteine, two leucine, two alanine and two arginine residues). Thus fraction 2 appears to differ from fraction 1 by seven residues per polypeptide chain at the N-terminus as depicted in Fig. 3.

Auto-antigenicity of the subfragments

The expression of certain allotypic markers and the 'general' rheumatoid factor auto-antigenic determinant on the thermolysin subfragments was examined. Fractions 1 and 2 expressed both the

Table 5. The capacity of the various thermolysin subfragments of the Fc fragment of human IgG1 to inhibit the agglutination, by pooled rheumatoid serum, of baboon IgG-sensitized erythrocytes. IgG and Fc and pFc′ fragments were employed as positive and negative controls respectively

Sample	Lowest concentration (mg/ml) of protein or fragment giving inhibition
Papain Fc	0·15
Fr. 1	0·30
Fr. 2	0·30
Fr. 3	> 5·00
Fr. 4A	> 5·00
Fr. 4B	> 5·00
Fr. 4C	> 5·00
pFc′	> 5·00

Gm(a) and Gm(x) markers in contrast to fraction 3, which only expressed the Gm(x) marker. A pepsin pFc′ fragment, which was included as a positive control, exhibited both Gm(a) and Gm(x) markers. The capacity of all the subfractions to react with 'general' rheumatoid factors is summarized in Table 5. Fractions 1 and 2 retained the ability to react with 'general' rheumatoid factors in contrast to fraction 3 and the three subfractions of fraction 4, which were unreactive.

DISCUSSION

Cleavage of the IgG1 Fc fragment by thermolysin was found to produce two major fragments: one containing the major part of the Fc fragment but devoid of the hinge region (fraction 2, designated tlmFc), the other corresponding to the Cγ3 domain (fraction 3, designated tlFc′). The tlmFc fragment appeared to consist of residues 235–446 of the γ chain, i.e. similar in size to the mFc fragment of Utsumi (1969), produced by papain cleavage of reduced and alkylated rabbit IgG. In addition, the tlmFc fragment appeared to be completely identical immunologically to the parent Fc fragment as did the mFc fragment of Utsumi (1969). The tlFc′ fragment showed complete identity with the pFc′ and tFc′ (Cγ3 domain) fragments on gel diffusion against sheep or guinea-pig antisera, but differed from these fragments with respect to expression of allotypic markers: the tlFc′ fragment expressed only the Gm(x) marker, whereas the pFc′ and tFc′ fragments retained both the Gm(a) and Gm(x) markers (Turner, Komvopoulos, Bennich and Natvig, 1972).

In the absence of dissociating agents (i.e. in physiological buffers) the tlmFc fragment appeared to retain the native dimeric structure of the intact Fc fragment. This indicates that non-covalent forces are the main stabilizing forces within the Fc fragment. A similar conclusion was proposed by Stevenson and Dorrington (1970) for heavy chain dimers, which were shown to assume a dimeric form even after reduction and carboxymethylation. Thus it would appear that the hinge region of the IgG molecule is not required for the structural integrity of the Fc fragment. This view is supported by the apparent total retention of species antigenicity of the tlmFc fragment, as demonstrated by its complete identity with the Fc fragment on gel diffusion analysis and the retention of the Gm(a) and (x) allotypic markers.

Cleavage of the γ chain by thermolysin at peptide bonds N-terminal to leucine and isoleucine residues is consistent with the known specificity of this enzyme for peptide bonds N-terminal to long chain hydrophobic residues within other proteins (Matusbara *et al.*, 1966). Similarly, cleavage by thermolysin of peptide bonds N-terminal to cysteine and C-terminal to threonine has been reported (Ambler and Meadway, 1968; Matsubara and Sasaki, 1968); this is in agreement with the observed cleavage of the Thr–Cys bond at position 225–226. The production of a Cγ3 domain fragment by thermolysin is in accordance with the similarity in specificity between thermolysin and pepsin, which also produces a Cγ3 domain fragment (pFc′) from IgG. In addition, prolonged digestion of Fc by thermolysin caused further degradation of both the tlmFc and tlFc′ fragments, an effect similar to that exhibited by pepsin on the pFc′ fragment.

Haemagglutination–inhibition studies indicated that the 'general' rheumatoid factor reactive determinant was present on the tlmFc fragment but not on the tlFc′ fragment. This provides further evidence that the reactive determinant is not located within the Cγ3 domain, nor in the immediate vicinity of the hinge region of the IgG molecule. It would appear therefore that this determinant may be located in the Cγ2 domain of the molecule, but away from the hinge region. It has been suggested that there may be considerable interaction between the Cγ2 and Cγ3 domains (Charlwood and Utsumi, 1969; Ghose, 1972; Dorrington, Bennich and Turner, 1972). Thus it is possible that the 'general' rheumatoid factor-reactive determinant may be dependent for its expression on interaction between the Cγ2 and Cγ3 domains. The lack of reactivity of either the Facb or pFc′ fragments of rabbit IgG with 'general' rheumatoid factors suggests that this hypothesis may at least hold for rabbit IgG (Stewart, Smith and Stanworth, 1973).

The lack of expression of the Gm(a) allotypic marker on the tlFc′ fragment is of interest in view of previous evidence of the localization of this marker within the Cγ3 domain. However, as complete physicochemical characterization of this fragment was not undertaken no definite conclusions can be made as to the cause of this phenomenon.

The production of an Fc fragment devoid of the hinge region (tlmFc) may be of considerable use in

investigating the involvement of the hinge region in other biological activities of the IgG Fc fragment such as complement fixation, capacity to mediate passive cutaneous anaphylaxis and membrane transmission.

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