The Products from Papain and Pepsin Hydrolyses of Guinea-Pig Immunoglobulins γ_1 G and γ_2 G

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1. The products from papain and pepsin hydrolyses of the guinea-pig immunoglobulins γ_1 G and γ_2 G were isolated and characterized with regard to molecular weight, amino acid composition, hexose content and antigenic specificity. 2. Fragments Fab and (Fab')₂ from immunoglobulins γ_1 G and γ_2 G have similar electrophoretic and antigenic properties, but show some class-specific differences in amino acid composition. 3. Three Fc fragments were obtained after papain digestion of immunoglobulin γ_2 G, namely, fragment Fc dimer (mol.wt. 58000), fragment Fc monomer (mol.wt. 29000) and fragment Fc' (mol.wt. 8000). A single crystalline fragment, namely fragment Fc' (mol.wt. 11000), was isolated after papain digestion of immunoglobulin γ_1 G. 4. Peptic digestion of immunoglobulins γ_1 G and γ_2 G releases C-terminal fragments, namely, fragments pFc', of similar molecular weight (13000) but different amino acid compositions and distinct antigenic specificities. 5. Digestion-time studies show that immunoglobulin γ_1 G is far more susceptible to proteolysis than is immunoglobulin γ_2 G and suggest that at least a proportion of molecules are split primarily at a site that liberates fragment γ_1 Fc'.

Digestion of the guinea-pig immunoglobulins γ_1 G and γ_2 G with papain results in the release of Fab and Fc fragments (Nussenzweig & Benacerraf, 1964). The γ_1 - and γ_2 -Fc fragments differ in antigenic specificity, electrophoretic mobility (Nussenzweig & Benacerraf, 1964), tryptic peptide 'maps' (Lamm, Lisowka-Bernstein & Nussenzweig, 1967) and amino acid composition (Lamm, 1969), whereas the Fab fragments from immunoglobulins γ_1 G and γ_2 G are indistinguishable by these criteria. Pepsin digestion of immunoglobulin γ_2 G produces a (Fab')₂ fragment that is antigenically identical with fragment Fab from either immunoglobulin γ_1 G or immunoglobulin γ_2 G (Nussenzweig & Benacerraf, 1966).

Many class-specific biological activities of immunoglobulins γ_1 G and γ_2 G (Ovary, Benacerraf & Bloch, 1963; Strejan & Campbell, 1967, 1968; Berken & Benacerraf, 1967; Bloch, Ovary, Kourilsky & Benacerraf, 1963; Osler, Oliveira, Shint & Sandberg, 1969) appear to arise from structural differences located in the Fc regions of their respective heavy chains. An attempt is being made in this laboratory to study the structural basis of certain of these biological activities. As a basis for this, immunoglobulins γ_1 G, γ_2 G and γ M and their constituent peptide chains have been isolated and

characterized (Leslie & Cohen, 1970a). Studies on the products from proteolysis of immunoglobulins γ_1 G and γ_2 G are reported in the present paper.

MATERIALS AND METHODS

Serum was collected by cardiac puncture from outbred guinea pigs (300–500 g) immunized with bovine immunoglobulin G, and immunoglobulins γ_1 G and γ_2 G were isolated from pooled immune sera as described by Leslie & Cohen (1970a).

Enzyme digestion. Papain digestion of immunoglobulins γ_1 G and γ_2 G was performed as described by Nussenzweig & Benacerraf (1964). A 1–2% solution of immunoglobulin in 0.1 M-NaCl–0.1 M-sodium phosphate buffer, pH 7.5, containing 10 mM-cysteine and 2 mM-EDTA, was incubated with 2% (w/w) papain (twice-crystallized; 10 units/mg; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) at 37°C for 4 h. The digestion was stopped by addition of iodoacetamide (final concentration, 11 mM). Pepsin digestion was carried out on 1–2% solutions of immunoglobulins γ_1 G and γ_2 G in 0.1 M-sodium acetate buffer, pH 4.5, with 2% (w/w) pepsin (blood-group-A-free; Mann Research Laboratories, New York, N.Y., U.S.A.) at 37°C for 20 h (Mandy, Rivers & Nisonoff, 1961). Digestion was stopped by raising the pH to 7 with 0.1 M-NaOH. In digestion-time studies (see the Results section) conditions were the same as those described above except that the amount of enzyme added was 1% by weight of protein.

Separation of fragments. Papain digests of immunoglobulin γ_2 G were equilibrated with 50 mM-tris-HCl buffer, pH 8.2, by passage through Sephadex G-25 and

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separated by DEAE-cellulose (Whatman DE22) chromatography. Fragment γ_2 Fab was recovered in the initial eluting buffer (50 mM-tris-HCl buffer, pH 8.2) and Fc fragments were eluted with a concentration gradient of 0–0.4 M-NaCl in 50 mM-tris-HCl buffer, pH 8.2, obtained by using a constant-volume mixing chamber (500 ml). The Fab fraction was separated from undigested immunoglobulin γ_2 G by passage through a Sephadex G-200 column (3.3 cm $\times$ 140 cm) in 0.1 M-NaCl–50 mM-tris-HCl buffer, pH 8.2. The Fc fragments were purified by gel filtration through Sephadex G-200 in 5 M-guanidinium chloride by using a column (2.5 cm $\times$ 100 cm) adapted for upward flow. These samples were treated before filtration with 5 mM-iodoacetamide to minimize disulphide interchange and were followed on to the column by 5–10% of the column volume of 5.5 M-guanidinium chloride (Small & Lamm, 1966). Peptic digestion products of immunoglobulin γ_2 G were dialysed against 0.1 M-NaCl–50 mM-tris-HCl buffer, pH 8.2, and separated by passage through Sephadex G-200 in the same buffer.

Papain or pepsin digests of immunoglobulin γ_1 G were dialysed against 2 mM-sodium phosphate buffer, pH 7.5, and the square plate-like crystals that formed were harvested and dissolved in 0.1 M-acetic acid. The supernatant from the peptic digestion containing γ_1 (Fab')₂ fragments was passed through Sephadex G-200 in 0.1 M-NaCl–50 mM tris-HCl buffer, pH 8.2. The supernatant from the papain digest of immunoglobulin γ_1 G was dialysed against 50 mM-tris-HCl buffer, pH 8.2, and eluted stepwise from DEAE-cellulose successively with 50 mM-tris-HCl buffer, pH 8.2, 50 mM-NaCl–50 mM-tris-HCl buffer, pH 8.2, and 0.2 M-NaCl–50 mM-tris-HCl buffer, pH 8.2.

Reduction of disulphide bonds. Immunoglobulin preparations were mildly reduced with 0.3 M- β -mercaptoethanol in 0.5 M-tris-HCl buffer, pH 8 (Fleischman, Porter & Press, 1963). Complete reduction of inter- and intra-chain disulphide bridges was carried out as described by Small & Lamm (1966) by using either 0.3 M- β -mercaptoethanol or 0.1 M-dithiothreitol. Thiol groups were blocked with iodoacetamide.

Determinations of molecular weights. Molecular weights of Fc fragments from immunoglobulins γ_1 G and γ_2 G were determined by gel filtration of totally reduced material through Sephadex G-200 in 5 M-guanidinium chloride (Cebra & Small, 1967). Replicate determinations were made. The first column employed was standardized with human immunoglobulin γ M, and fully reduced samples of rabbit γ -chain, rabbit light chain and ¹³¹I-labelled ribonuclease; the second column was standardized with ¹³¹I-labelled guinea-pig immunoglobulin γ M, and fully reduced guinea-pig μ -chain, γ_2 -chain, light chain and ¹³¹I-labelled lysozyme. Proteins were labelled with ¹³¹I by the method of McFarlane (1958).

Amino acid analyses. These were performed on a Locarte analyser as described by Leslie & Cohen (1970a). Cysteine was determined as *S*-carboxymethylcysteine from totally reduced fragments, and tryptophan was measured spectrophotometrically (Melamed & Green, 1963).

Hydrazinolysis. This was performed by the method of Bradbury (1956) and Fraenkel-Conrat & Tsung (1967). The dry peptide (0.5 μ mol), hydrazine sulphate (26 mg) and hydrazine (0.2 ml; Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) were sealed in an ampoule under

vacuum. After 16 h at 60°C the excess of hydrazine was removed *in vacuo* and the hydrazides were extracted with benzaldehyde. The aqueous phase was taken to dryness, the residue dissolved in 0.2 M-sodium citrate buffer, pH 2.2, and the free amino acids were determined on the analyser.

Determination of hexose. Hexose was determined by a micro-modification of the method described by Winzler (1955).

Antisera. Antisera against guinea-pig serum immunoglobulin γ_1 G and immunoglobulin γ_2 G were raised in outbred rabbits (2–3 kg). Animals were injected on days 1 and 8 with approx. 2 mg of antigen in Freund's complete adjuvant at multiple subcutaneous sites and then boosted on day 21 with an intraperitoneal injection of alumina-adsorbed antigen (3 mg) in 0.9% NaCl suspension. Bleedings were made from an ear vein on days 28 and 35. Antisera raised against immunoglobulin γ_1 G or immunoglobulin γ_2 G reacted strongly with their respective Fc fragments and with Fab fragments from either class (Thorbecke, Benacerraf & Ovary, 1963; Nussenzweig & Benacerraf, 1964). The anti-(immunoglobulin γ_1 G) serum also contained some antibodies against γ_2 Fc fragments and transferrin. Anti-(immunoglobulin γ_2 G) serum was rendered class-specific by absorption with immunoglobulin γ_1 G. Similarly, anti-(immunoglobulin γ_1 G) serum was absorbed with immunoglobulin γ_2 G and with guinea-pig serum from which the immunoglobulins had been removed by precipitation in 50%-saturated (NH₄)₂SO₄.

Other methods. Immunoelectrophoresis was performed by the method of Scheidegger (1955). Immunodiffusion was carried out according to the method of Ouchterlony (1953). Starch-gel electrophoresis was performed by the method of Edelman & Poulik (1961).

RESULTS

Products of papain digestion

Hydrolysis of immunoglobulin γ_2 G. Digestion of immunoglobulin γ_2 G at 37°C for 4 h with papain generated three electrophoretically distinct components (Plate 1b) separable by chromatography on DEAE-cellulose (Plate 1a). Fragment Fab, which was eluted with the initial buffer, contained a trace of undigested immunoglobulin γ_2 G, which was removed by gel filtration through Sephadex G-200. The Fc fragments were isolated as three pools after gradient elution of the DEAE-cellulose column. The first and second pools (Plate 1a) contained fragment γ_2 Fc contaminated respectively with fragment Fab and an Fc fragment relatively deficient in antigenic determinants. The third peak contained only the antigenically deficient fragment (Fc').

All three gradient pools were subjected to gel filtration through Sephadex G-200 in 5 M-guanidinium chloride. The first was eluted as a major peak in the position of totally reduced γ -chains, indicating a molecular weight of about 50 000. The second was resolved into three distinct peaks (Plate 2a), having *K*_D values approximating those

of γ -chains, light chains and the Fc' fragment present in gradient pool III respectively. The two larger Fc fragments were antigenically indistinguishable and carried all the class-specific γ_2 determinants, whereas the third fraction was antigenically deficient and showed a reaction of identity with fragment Fc' from DEAE-cellulose chromatography (Plate 2b). Total reduction of gradient pool II resulted in the elimination of the first Sephadex G-200 peak and a corresponding increase in the size of the second peak.

It therefore appears that these conditions of papain digestion produce whole fragment γ_2 Fc in both dimeric (Fc dimer) and monomeric (Fc monomer) forms and also a smaller antigenically deficient fragment (Fc'). The fragment Fc dimer is converted into the monomer after reduction.

The recovery in extinction units of fragment Fab is 59% of the starting material, which approximates the theoretical value (61.5%). The major loss as small peptides arises from papain degradation of the Fc region (Plate 1a). The relative proportions of the three Fc fragments are shown in Plate 2(a).

Hydrolysis of immunoglobulin γ_1 G. Dialysis of the products of papain digestion of immunoglobulin γ_1 G against 2mM-phosphate buffer, pH 7.5, produces a crystalline Fc' fragment, sparingly soluble at neutral pH (Nussenzweig & Benacerraf, 1964). This Fc' fragment is antigenically deficient to whole immunoglobulin γ_1 G, as shown by Lamm *et al.* (1967), and on passage through Sephadex G-200 in 5M-guanidinium chloride is eluted as a single peak substantially later than totally reduced light chains. The fragment is designated in this paper as fragment γ_1 Fc'.

The soluble portion of the papain digest of immunoglobulin γ_1 G contained one fraction that carried only Fab determinants and a second component of faster electrophoretic mobility with γ_1 -class-specific determinants. Some separation of these components was achieved by stepwise elution from DEAE-cellulose with successively 0M-, 50mM- and 0.2M-sodium chloride in 50mM-tris-HCl buffer, pH 8.2, but fragment γ_1 Fab was present in all three

fractions. The fragment Fc material that was recovered as a late peak with the second buffer carried all the class-specific determinants and was similar in size to fragment γ_2 Fc dimer on filtration through Sephadex G-200 in 5M-guanidinium chloride. This fragment was contaminated with fragment Fab and recovery was less than 7% of the starting material, which probably represents only 10–20% of the total Fc fragment. The yield of γ_1 Fc' fragment was about 11% from a 4h papain digest.

Products of pepsin digestion

Hydrolysis of immunoglobulin γ_2 G. Pepsin digestion of immunoglobulin γ_2 G produced two major fragments with identical electrophoretic mobilities. These were separated by passage through Sephadex G-200 in 50mM-tris-HCl buffer, pH 8.2 (Plate 3a), yielding a major component [fragment (Fab')₂] antigenically indistinguishable from fragment Fab, and a smaller fragment (pFc'), which carried the same class-specific determinants as the papain γ_2 Fc' fragment (Plate 3b). Overall recoveries were 54% for fragment (Fab')₂ and 14% for fragment γ_2 pFc'.

Hydrolysis of immunoglobulin γ_1 G. Neutralization of a 20h pepsin digest of immunoglobulin γ_1 G leads to formation of square crystals (fragment γ_1 pFc') similar to those obtained by papain digestion and present in slightly higher yield (14% of starting material). The fragment γ_1 pFc', on gel filtration through Sephadex G-200 in 5M-guanidinium chloride, was eluted somewhat earlier than was fragment γ_1 Fc' in a position almost identical with that of fragment γ_2 pFc'. The fragment γ_1 pFc' displays a reaction of identity with fragment γ_1 Fc' from papain hydrolysis when analysed by double diffusion against γ_1 -class-specific antiserum (Plate 4a).

The soluble pepsin digest contained only Fab determinants and showed a single arc on immunoelectrophoresis against anti-(immunoglobulin γ_1 G) serum. This fragment was eluted from Sephadex

Table 1. *Molecular weights of fragments from guinea-pig immunoglobulins γ_1 G and γ_2 G*

The column of s.d. values includes s.d. in calculation of best straight-line fit of data and s.d. for K_D determinations of unknowns.

Fragment	No. of determinations	Molecular weight	
		Mean	s.d.
γ_2 Fc monomer	3	28 900	2100
γ_2 Fc'	2	7750	1060
γ_2 pFc'	3	13 000	410
γ_1 Fc'	3	10 600	1200
γ_1 pFc'	3	13 000	390

G-200 in 50mm-tris-HCl buffer, pH 8.2, as a single peak in the same position as fragment $\gamma_2(\text{Fab}')_2$.

As reported by Nussenzweig & Benacerraf (1964, 1966), Fab and $(\text{Fab}')_2$ fragments from immunoglobulins $\gamma_1\text{G}$ or $\gamma_2\text{G}$ were all indistinguishable when made to react with unabsorbed anti-(immunoglobulin $\gamma_1\text{G}$) antiserum.

Properties of the fragments

Molecular weights. Molecular-weight determinations (Table 1) were performed on standardized columns and plots made of $\sqrt{K_D}$ versus $\sqrt{\text{molecular weight}}$.

The elution peaks of all fragments examined ($\gamma_2\text{Fc}$ monomer, $\gamma_2\text{Fc}'$, $\gamma_1\text{Fc}'$, $\gamma_2\text{pFc}'$ and $\gamma_1\text{pFc}'$) were advanced rather than retarded after total reduction, indicating that all are single polypeptide chains probably possessing one or more intrachain disulphide loops. The $\gamma_2\text{Fc}$ monomer fragment (mol.wt. 29000 ± 2000) accounts for 55% of the γ_2 -chain (mol.wt. 53000; Leslie & Cohen, 1970a) so that, even if papain causes a single split in the γ_2 -chain, the molecular weight of the *N*-terminal Fd fragment cannot exceed 24000 ± 3000 . Reduced fragment $\gamma_2\text{Fc}$ dimer is eluted in the same position as fragment Fc monomer, indicating a molecular weight for the dimer of 58000. Fragment $\gamma_2\text{Fc}'$

(mol.wt. 8000 ± 1000) represents less than one-third of the whole Fc fragment. Fragment $\gamma_1\text{Fc}'$ (mol.wt. 11000 ± 1000) is not analogous to fragment $\gamma_2\text{Fc}'$ since its molecular weight is about 50% greater. The peptic fragments $\gamma_1\text{pFc}'$ and $\gamma_2\text{pFc}'$ are almost identical in size (mol.wt. 13000 ± 400) and may represent analogous portions of the γ -chains.

Amino acid analysis. The amino acid compositions of all fragments except fragment $\gamma_2\text{Fc}$ dimer were determined. All fragments except fragments $\gamma_1\text{Fab}$, $\gamma_1\text{Fc}'$ and $\gamma_1\text{pFc}'$ were purified before analysis by gel filtration through Sephadex G-200 in either 0.1M-NaCl-50mm-tris-HCl buffer, pH 8.2, or 5M-guanidinium chloride; fragments $\gamma_1\text{Fc}'$ and $\gamma_1\text{pFc}'$ were recrystallized twice before analysis, and fragment $\gamma_1\text{Fab}$ was recovered from the first step of DEAE-cellulose chromatography. The present results (Table 2) on $\gamma_1\text{Fab}$ and $\gamma_2\text{Fab}$ fragments support an earlier observation (Lamm, 1969) that their amino acid compositions are very similar. Only four residues display significant (>10%) differences; these are alanine and methionine (high content in fragment $\gamma_2\text{Fab}$) and histidine and proline (high content in fragment $\gamma_1\text{Fab}$). Differences in methionine and histidine, but not in alanine or proline, were also observed between the Fab fragments from anti-2,4-dinitrophenol anti-

Table 2. Amino acid compositions of Fab and $(\text{Fab}')_2$ fragments and of $\gamma_2\text{Fc}$ monomer

Residue	Amino acid composition (mol of residue/100 mol of residues)				
	Fragment Fab from		Fragment $(\text{Fab}')_2$ from		Fragment $\gamma_2\text{Fc}$ monomer
	Immunoglobulin $\gamma_1\text{G}$	Immunoglobulin $\gamma_2\text{G}$	Immunoglobulin $\gamma_1\text{G}$	Immunoglobulin $\gamma_2\text{G}$	
Lys	4.88	4.75	4.29	4.56	8.10
His	1.63	1.39	1.57	1.41	2.26
Arg	3.38	3.64	3.17	3.47	3.65
Asx	7.25	6.93	7.36	7.33	9.58
Thr	9.41	9.47	9.46	9.57	7.19
Ser	13.7	13.7	13.6	12.7	7.79
Glx	8.64	8.86	8.98	8.83	10.2
Pro	6.31	5.66	5.99	6.92	9.73
Gly	8.06	7.91	8.31	8.11	3.99
Ala	6.05	6.86	5.97	6.29	4.87
Val	7.71	7.66	7.34	7.56	10.0
Met	1.09	1.20	1.06	1.27	1.37
Ile	3.51	3.49	3.55	3.37	4.30
Leu	7.09	7.38	7.07	7.28	5.51
Tyr	4.45	4.44	4.17	3.86	3.22
Phe	2.84	3.07	2.92	3.04	4.03
Trp	2.26	2.12	1.93	1.75	1.97
Cys (a)*	2.04	2.15	3.07	2.87	2.36
(b)†	0.436	0.404			

* From total reduction of all disulphide bonds.

† From mild reduction of susceptible disulphide bonds (see the Materials and Methods section).

EXPLANATION OF PLATE I

(a) DEAE-cellulose chromatography of a papain digest of immunoglobulin $\gamma_2\text{G}$. The initial buffer was 50 mM-tris-HCl buffer, pH 8.2, and a 0.4 M-NaCl gradient in 50 mM-tris-HCl buffer, pH 8.2, was used. The flow rate was 25–30 ml/h. Arrows indicate fractions pooled and the values given are the percentages of the original protein recovered in each pool. (b) Immunoelectrophoresis of fragments from papain digestion of immunoglobulin $\gamma_2\text{G}$. Well 1, immunoglobulin $\gamma_2\text{G}$; well 2, 4 h digest of immunoglobulin $\gamma_2\text{G}$; well 3, pre-gradient pool (fragment Fab); well 4, gradient pool I; well 5, gradient pool II; well 6, gradient pool III (fragment Fc'). The troughs contained: (i) rabbit anti-(immunoglobulin $\gamma_2\text{G}$); (ii) class-specific anti-(γ_2 -chain) serum.

EXPLANATION OF PLATE 2

(a) Gel filtration of gradient pool II (Plate 1a) of a papain digest of immunoglobulin γ_2 G in 5 M-guanidinium chloride on a column (2.54 cm $\times$ 100 cm) of Sephadex G-200 adapted for upward flow. The flow rate was 10–15 ml/h; 5 ml fractions were collected. Percentage recoveries of the original protein are given below each peak. (b) Double immunodiffusion of Fc fragments. Wells 1 and 2, immunoglobulin γ_2 G; well 3, fragment Fc dimer (peak 1, Plate 2a); well 4, fragment Fc monomer (peak 2, Plate 2a); well 5, fragment Fc' (peak 3, Plate 2a); well 6, fragment Fc' from DEAE-cellulose chromatography (gradient pool III, Plate 1a). The centre well contained class-specific anti-(γ_2 -chain) serum.

EXPLANATION OF PLATE 3

(a) Gel filtration of a pepsin digest of immunoglobulin $\gamma_2\text{G}$ through Sephadex G-200 in 0.1 M-NaCl-50 mM-tris-HCl buffer, pH 8.2. The flow rate was 10–15 ml/h; 10 ml fractions were collected. Arrows indicate elution peaks of marker proteins. (b) Double immunodiffusion of Fc' fragments. Well 1, immunoglobulin $\gamma_2\text{G}$; well 2, fragment $\gamma_2\text{pFc}'$; well 3, fragment $\gamma_2\text{Fc}'$. The centre well contained class-specific anti-(γ_2 -chain) serum.

EXPLANATION OF PLATE 4

(a) Double immunodiffusion of $\gamma_1\text{Fc'}$ fragments. Wells 1 and 2, immunoglobulin $\gamma_1\text{G}$; well 3, fragment $\gamma_1\text{pFc'}$ (peptic digest); well 4, fragment $\gamma_1\text{Fc'}$ (papain digest). The centre well contained class-specific anti- $(\gamma_1\text{-chain})$ serum. (b) Immunoelectrophoresis of papain digests of immunoglobulin $\gamma_2\text{G}$ after different time-intervals. Well 1, undigested immunoglobulin $\gamma_2\text{G}$; well 2, $\frac{1}{2}$ h digest; well 3, 1 h digest; well 4, 2 h digest; well 5, 4 h digest; well 6, 8 h digest; well 7, 16 h digest. The troughs contained anti- $(\gamma_2\text{-chain})$ serum.

EXPLANATION OF PLATE 5

Urea-formate starch-gel electrophoresis of papain digests of immunoglobulin γ_1 G. Electrophoresis was carried out for 18 h at 180 V and 26 mA. Staining was with Amido Black. Slot 1, undigested immunoglobulin γ_1 G; slot 2, 1 min digest; slot 3, 2 min digest; slot 4, 5 min digest; slot 5, 10 min digest; slot 6, 20 min digest; slot 7, 40 min digest; slot 8, 1 h digest; slot 9, 2 h digest; slot 10, 16 h digest; slot 11, fragment γ_1 Fab; slot 12, undigested immunoglobulin γ_1 G. Arrows indicate identified fragments.

EXPLANATION OF PLATE 6

Immunoelectrophoresis of papain digests of immunoglobulin γ_1 G. Well 1, immunoglobulin γ_1 G; well 2, 1 min digest; well 3, 2 min digest; well 4, 5 min digest; well 5, 10 min digest; well 6, 20 min digest; well 7, 40 min digest. The left-hand troughs contained anti-(immunoglobulin γ_1 G) serum and the right-hand troughs contained class-specific anti-(γ_1 -chain) serum.

EXPLANATION OF PLATE 7

Urea-formate starch-gel electrophoresis of peptic digests of immunoglobulin γ_2 G. Electrophoresis and staining were as described in Plate 5. Slot 1, undigested immunoglobulin γ_2 G; slot 2, fragment $\gamma_2(\text{Fab}')_2$; slot 3, 1 h digest; slot 4, 2 h digest; slot 5, 4 h digest; slot 6, 8 h digest; slot 7, 20 h digest; slot 8, fragment $\gamma_2\text{pFc}'$.

EXPLANATION OF PLATE 8

Urea-formate starch-gel electrophoresis of pepsin digests of immunoglobulin γ_1 G. Electrophoresis and staining were as described in Plate 5. Slot 1, immunoglobulin γ_1 G; slot 2, fragment $\gamma_1(\text{Fab}')_2$; slot 3, 1 h digest; slot 4, 2 h digest; slot 5, 4 h digest; slot 6, 8 h digest; slot 7, 20 h digest; slot 8, fragment $\gamma_1\text{pFc}'$.

Table 3. Comparison of the amino acid compositions of fragments γ_2 Fc and γ_2 Fab with that of immunoglobulin γ_2 G

Residue	Amino acid composition (mol of residue/mol of fragment)			
	Fragment γ_2 Fab	Fragment γ_2 Fc	Fragments $2\gamma_2$ Fab + γ_2 Fc (calculated)	Immunoglobulin γ_2 G§
Lys	20.2	39.9	80.3	80.4
His	5.9	11.1	22.9	22.7
Arg	15.5	17.8	48.8	45.0
Asx	29.4	46.9	105	104
Thr	40.2	35.2	116	116
Ser	57.6	38.2	153	153
Glx	37.6	49.1	124	122
Pro	24.0	47.7	95.7	104
Gly	33.5	19.6	86.6	86.0
Ala	29.1	23.8	82.0	77.4
Val	32.5	49.0	114	113
Met	5.1	6.7	16.9	17.4
Ile	14.8	21.1	50.7	50.8
Leu	31.3	27.0	89.6	90.0
Tyr	18.7	15.8	53.2	53.2
Phe	13.0	19.8	45.8	44.9
Trp	9.1	9.5	27.7	30.1
Cys	9.1	11.6	29.8	34.5
Total*	427†	490†	1344	1344

* Based on the estimation of total residues/fragment, assuming no amino acid losses in digestion and carbohydrate on fragment Fc only. Carbohydrate content is 4400 g/mol of immunoglobulin γ_2 G (Leslie & Cohen, 1970a).

† Mol.wt. of fragment γ_2 Fab = $\frac{1}{2} \times 152\,000$ (mol.wt. of immunoglobulin γ_2 G) – 29 000 (mol.wt. of Fc monomer) = 47 000. No. of residues/mol of fragment Fab = 47 000/100 = 427.

‡ Mol.wt. of carbohydrate-free fragment Fc = $2 \times 29\,000 - 4400 = 53\,600$. No. of residues/mol of fragment Fc = 53 600/110 = 488 $\approx$ 490.

§ Leslie & Cohen (1970a).

bodies (Lamm, 1969). The γ_1 (Fab')₂ and γ_2 (Fab')₂ fragments are also similar. As with the Fab fragments, fragment γ_1 (Fab')₂ contains less methionine and more histidine than does fragment γ_2 (Fab')₂; the proline content, on the other hand, is higher in fragment γ_2 (Fab')₂. Compared with fragment γ_2 Fab, the γ_2 (Fab')₂ fragment is richer in proline and half-cystine and contains less serine, alanine and tyrosine. This suggests the presence in the peptic fragments (Fab')₂ of a proline-rich hinge region in the vicinity of the interchain disulphide bridges, as occurs in rabbit and human immunoglobulins γ G (Smyth & Utsumi, 1967; Steiner & Porter, 1967; Edelman *et al.* 1969). By contrast, the proline contents of fragments γ_1 (Fab')₂ and γ_1 Fab are not significantly different, though the half-cystine is higher in the former. This suggests different sites of enzymic cleavage for immunoglobulins γ_1 G and γ_2 G indicating class-specific differences in the hinge region.

Comparison of the present analyses of γ_1 - and γ_2 -Fab fragments with those of anti-2,4-dinitro-

phenol antibodies (Lamm, 1969) reveals that the former fragments contain more histidine and methionine and less serine than do the latter antibody fragments. In addition, the present γ_1 Fab fragment is richer in lysine, proline and isoleucine, and fragment γ_2 Fab contains less half-cystine.

Analyses of the Fc fragments show that (i) fragments γ_2 Fab and γ_2 Fc monomer are chemically distinct (Table 2); (ii) γ_1 Fc fragments analysed in this study and anti-2,4-dinitrophenol preparations (Lamm, 1969) are similar and only the serine content differs by more than 1 mol of residue/100 mol. This probably represents a systematic difference in analyses since the serine content is higher in almost all the analyses of anti-2,4-dinitrophenol antibodies and that of methionine is higher in our preparations; (iii) the calculated amino acid content of the fragments 2(Fab + Fc monomer) agrees closely with that of the whole immunoglobulin (Table 3). Only the half-cystine contents display a significant difference (>10%).

The pFc' fragments from immunoglobulins γ_1 G

Table 4. *Amino acid compositions of Fc' and pFc' fragments*

The compositions are normalized to a mean value of 2 mol of residue/mol of fragment for methionine, tryptophan and half-cystine. Nearest whole numbers are given in parentheses.

Residue	Amino acid composition (mol of residue/mol of fragment)			
	Fragment $\gamma_1\text{pFc}'$	Fragment $\gamma_1\text{Fc}'$	Fragment $\gamma_2\text{pFc}'$	Fragment $\gamma_2\text{Fc}'$
Lys	8.12 (8)	6.76 (7)	9.48 (9)	5.35 (5)
His	2.04 (2)	1.40 (1)	4.00 (4)	2.10 (2)
Arg	4.60 (5)	3.97 (4)	4.22 (4)	2.71 (3)
Asx	12.7 (13)	12.8 (13)	11.3 (11)	9.30 (9)
Thr	7.65 (8)	5.69 (6)	8.69 (9)	5.79 (6)
Ser	13.4 (13)	11.0 (11)	12.4 (12)	8.87 (9)
Glu	8.75 (9)	8.30 (8)	9.27 (9)	7.35 (7)
Pro	12.6 (13)	11.9 (12)	10.7 (11)	7.46 (7)
Gly	4.26 (4)	4.20 (4)	4.15 (4)	2.77 (3)
Ala	4.26 (4)	3.76 (4)	7.15 (7)	5.47 (5)
Val	7.18 (7)	6.76 (7)	10.8 (11)	8.16 (8)
Met	1.96 (2)	1.95 (2)	1.93 (2)	1.64 (2)
Ile	6.01 (6)	4.90 (5)	5.35 (5)	3.58 (4)
Leu	7.12 (7)	6.76 (7)	6.34 (6)	5.37 (5)
Tyr	3.85 (4)	3.93 (4)	5.11 (5)	4.17 (4)
Phe	4.96 (5)	4.82 (5)	3.32 (3)	2.80 (3)
Trp	2.15 (2)	2.42 (2)	2.02 (2)	2.12 (2)
Cys	1.91 (2)	1.72 (2)	2.04 (2)	1.87 (2)
Total	113.5 (114)	101.2 (104)	118.0 (116)	86.9 (86)
Mol.wt. (a)*	12485 (12540)	11132 (11440)	12980 (12760)	9559 (9460)
(b)†	13000 $\pm$ 400	10600 $\pm$ 1200	13000 $\pm$ 400	7750 $\pm$ 1100

* Estimated from amino acid composition, assuming mean residue weight of 110.

† Estimated from gel filtration (see the Results section).

and $\gamma_2\text{G}$ have very similar molecular weights but different amino acid compositions (Table 4). Calculation of compositions (mol of residues/mol) for the Fc' fragments assuming 2 mol of residues/mol for half-cystine, tryptophan and methionine gives molecular weights closely corresponding to measured values (Table 4). This facilitates a comparison between Fc' fragments from papain and pepsin digestion, and shows that fragments $\gamma_1\text{pFc}'$ and $\gamma_1\text{Fc}'$ are closely similar in composition, a finding consistent with their antigenic identity. The findings suggest that fragment $\gamma_1\text{pFc}'$, compared with the smaller fragment $\gamma_1\text{Fc}'$, contains lysine, histidine, arginine, threonine(2), serine(2), proline and isoleucine as additional residues. The pFc' and Fc' fragments from immunoglobulin $\gamma_2\text{G}$ are not of comparable size and display greater differences in composition. However, fragment $\gamma_2\text{pFc}'$ does contain all the residues present in fragment $\gamma_2\text{Fc}'$. The ratio of acidic to basic amino acids is greater in fragment $\gamma_2\text{Fc}'$ than in fragment $\gamma_2\text{pFc}'$, in keeping with the greater electrophoretic mobility of the former.

Hexose content. The observation (Berken & Benacerraf, 1967) that hexose is confined mainly to fragment Fc was borne out in the present work. The

Fab and (Fab')₂ fragments possess less than 10% of the carbohydrate found in fragment $\gamma_2\text{Fc}$ monomer and about 10% of that present in γ_2 -chain. Further, the Fc' fragments from immunoglobulins $\gamma_1\text{G}$ and $\gamma_2\text{G}$ are almost devoid of carbohydrate.

C-terminal residues. Analysis of C-terminal amino acid after hydrazinolysis yielded similar results for the whole γ_1 -chain and its fragments, $\gamma_1\text{Fc}'$ and $\gamma_1\text{pFc}'$ (Table 5), suggesting that these fragments are C-terminal in position. A similar result was obtained with the γ_2 -chain and its fragments, $\gamma_2\text{Fc}$ monomer and $\gamma_2\text{pFc}'$, suggesting that these are also C-terminal. The fragment $\gamma_2\text{Fc}'$, on the other hand, showed a distinct pattern of C-terminal residues (Table 5) indicating more than one site of papain cleavage.

Time studies of proteolysis

Papain digestion. The products of papain digestion of immunoglobulin $\gamma_2\text{G}$ after $\frac{1}{2}$ –16 h were examined by immunoelectrophoresis (Plate 4b) and starch-gel electrophoresis in 8M-urea-formate buffer, pH 3.5. It is apparent that the major products from immunoglobulin $\gamma_2\text{G}$ after $\frac{1}{2}$ –2 h are fragment Fab and whole fragment Fc, and that

Table 5. C-terminal amino acids recovered after hydrazinolysis of heavy chains and fragments

	Amino acid (% of total)						
	Immunoglobulin γ_2 G				Immunoglobulin γ_1 G		
	γ_2 -chain	Fragment γ_2 Fc monomer	Fragment γ_2 pFc'*	Fragment γ_2 Fc'	γ_1 -chain	Fragment γ_1 pFc'	Fragment γ_1 Fc'
Asp	8	14	11 (12)	7	7	10	13
Ser	16	14	21 (6)	7		11	
Glu				3			
Gly	62	57	60 (75)	30	76	79	75
Ala	7	15	8 (7)	19	7		
Val				9			
Met					10		
Ile				3			
Leu	7			22			12

* Values in parentheses show results on fragment γ_2 pFc' after filtration through Sephadex G-200 in 5 M-guanidinium chloride.

appreciable degradation of fragment Fc to fragment Fc' occurs only after 4–8 h. By 16 h whole fragment Fc is almost completely degraded and the Fc' fragment is substantially hydrolysed. Starch-gel electrophoresis supports these observations and, in addition, demonstrates that splitting of immunoglobulin γ_2 G is virtually complete after 1–2 h and that fragment Fab is highly resistant to further digestion.

By contrast, digestion of immunoglobulin γ_1 G to its final products is complete after only 1 h of papain digestion and little change occurs on more prolonged incubation (Plate 5). Shorter time-intervals examined by immunoelectrophoresis indicate that primary splitting of immunoglobulin γ_1 G takes place very rapidly (Plate 6). Starch-gel electrophoresis shows that formation of fragment Fc' occurs within 2 min. The fragments formed between 10 and 40 min have not been completely identified, but production of fragment Fab is evidently incomplete after 40 min. These results suggest that at least a proportion of the immunoglobulin γ_1 G molecules may be split primarily at a site that produces the Fc' fragment. The nature of the slow-mobility core fragment that persists after prolonged digestion (Plate 5) has not been established.

Peptic digestion. Peptic digestion of immunoglobulin γ_2 G proceeds very slowly and even after 20 h detectable amounts of undigested immunoglobulin are present (Plate 7). The γ_2 pFc' fragment appears within 1 h and reaches peak yields after 4–8 h, suggesting that more prolonged digestion results mainly in degradation to a homogeneous (Fab')₂ fragment. The material with mobility intermediate between those of fragments (Fab')₂ and

pFc' has not been identified and was not recovered after gel filtration of a 20 h pepsin digest.

Immunoglobulin γ_1 G is relatively susceptible to hydrolysis with pepsin. The final products are present after only 1 h and very little change occurred with more prolonged digestion except for a slight diminution in the amount of fragment pFc' after 20 h (Plate 8).

DISCUSSION

The present work confirms that γ_1 - and γ_2 -Fab fragments are very similar in antigenic and electrophoretic properties (Nussenzweig & Benacerraf, 1964) and in amino acid compositions (Lamm, 1969), and demonstrates that this similarity extends to the (Fab')₂ fragments isolated after peptic digestion. However, differences in the histidine and methionine contents of Fab fragments from immunoglobulins γ_1 G and γ_2 G occur in the fragments analysed in this study, and also those derived from anti-2,4-dinitrophenol antibodies (Lamm, 1969). This is in accord with the presence of class-specific determinants on the Fd fragments of guinea-pig Fab fragment (Leslie & Cohen, 1970b).

The Fc products from 4 h papain digestion of immunoglobulin γ_2 G are heterogeneous and can be separated into three distinct fragments: fragment Fc dimer (mol.wt. 58 000), fragment Fc monomer (mol.wt. 29 000) and fragment Fc' (mol.wt. 8000–9000). The fragment γ_2 Fc' is antigenically deficient with respect to the larger Fc fragments, which appear to represent the entire C-terminal half of the heavy chains. The only Fc fragment isolated from papain digests of immunoglobulin γ_1 G is fragment Fc' (mol.wt. 11 000). Its small size is consistent with

Fig. 1. Schematic representation of Fc fragments from immunoglobulin γ_2G and immunoglobulin γ_1G , giving their probable positions in the Fc regions (see the Discussion section).

the observation that it lacks antigenic determinants and several tryptic peptides characteristic of immunoglobulin γ_1G (Lamm *et al.* 1967).

The Fc products from pepsin digestion (fragments γ_1pFc' and γ_2pFc') display considerable differences in amino acid composition that reflect some of the differences between γ_1 - and γ_2 -chains (Leslie & Cohen, 1970a). Close similarity in the molecular weights of fragments γ_1pFc' and γ_2pFc' (both 13000) and the observation that both pFc' fragments possess the same C-terminal residues as the parent γ -chains suggest that they originate from equivalent positions (Fig. 1). The presence of 2 mol of half-cystine/mol of fragment pFc' indicates that both fragments γ_1pFc' and γ_2pFc' contain the C-terminal disulphide loop of the γ -chain and so resemble the pFc' fragment of human immunoglobulin γ_1G (Turner & Bennich, 1968). Heterogeneity of C-terminal residues observed with guinea-pig γ -chains has also been found in the goat and in both species may indicate the presence of subclasses of immunoglobulin γG (Givol & Hurwitz, 1969).

The γ_1Fc' and γ_2Fc' fragments, though smaller than the corresponding peptic fragments, are chemically and antigenically similar to the respective pFc' fragments and must originate from similar

regions of the γ -chains (Fig. 1). It is noteworthy that fragment γ_1Fc' , which is larger than fragment γ_2Fc' , yields the same C-terminal residues as the γ_1 -chain and therefore appears to be a C-terminal peptide. In this respect it differs from the Fc' fragments from guinea-pig γ_2 -chains and from human γ_1 -chains (Grey & Abel, 1967; Turner & Bennich, 1968).

The Fc' fragments from immunoglobulin γ_2G show greater antigenic deficiency with respect to whole Fc fragment than do the Fc' fragments from immunoglobulin γ_1G (Plates 3b and 4a). This fact, together with the relative resistance of γ_1 -chain to total reduction with β -mercaptoethanol in 7M-guanidinium chloride (R. G. Q. Leslie, unpublished work) and the inconsistency between molecular weights of immunoglobulins γ_1G and γ_2G as derived from ultracentrifugation and gel filtration (Leslie & Cohen, 1970a), suggests conformational differences between immunoglobulins γ_1G and γ_2G in the Fc regions. Such differences presumably account for the relatively greater susceptibility of immunoglobulin γ_1G to proteolysis by papain and pepsin.

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