Allotype-Related Sequence Variation of the Heavy Chain of Rabbit Immunoglobulin G

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The heavy chain of rabbit immunoglobulin G exists in three major allotypic patterns, Aa1–Aa3. A comparison of the amino acid compositions of the heavy chains isolated from immunoglobulin IgG homozygous for each allotypic determinant revealed the presence of an additional methionine residue per chain in the Aa3 allotype relative to the Aa1 and Aa2 allotypes. The position of the additional methionine residue was determined by cyanogen bromide cleavage and by tryptic digestion of the γ -chains; it coincided with the inter-Fd–Fc area of the chain. Isolation and characterization of the corresponding tryptic peptides of 31 amino acid residues from each of the allotypes showed the presence of a methionine-for-threonine replacement in the Aa3 allotype, but only in about 70–80% of the molecules. No other allotypic variations were seen in this tryptic peptide. Allotypically related variations in composition were also detected in the *N*-terminal cyanogen bromide-cleavage peptide.

The nomenclature employed here for rabbit immunoglobulin G (IgG) and its fragments follows that recommended by the World Health Organisation (1964).

It appears probable that the antibody activity of immunoglobulins is determined at least in part by their primary structure (Haber, 1964; Whitney & Tanford, 1965*a,b*). Thus it becomes important to distinguish those variations in amino acid sequence that are unrelated to the structural differences controlling antibody specificity. With rabbit IgG no clear division into subclasses has been found on the basis of antigenic properties or biological activity. It has been possible, however, to distinguish genetically segregating forms of rabbit IgG on the basis of their differing antigenicity, these distinctions being known as allotype specificities (Oudin, 1956; Dray, Dubiski, Kelus, Lennox & Oudin, 1962). The six major allotypic specificities or patterns, A1–A6, are controlled by six genes distributed into two series of three alleles, the alleles A1, A2 and A3 being associated with the *a* locus, and A4, A5 and A6 with the *b* locus. It has since been demonstrated that the specificities of the *a* locus are located on the γ -chain and those of the *b* locus on the light chain (Kelus, 1963;

Oudin, 1963; Sternke, 1964). Earlier investigators (Kelus, Marrack & Richards, 1961; Marrack, Richards & Kelus, 1962) had established that the specificities of both loci were present in the Fab fragments; hence the *a* locus controls structures in the Fd portion of the heavy chain.

The use of cyanogen bromide to cleave the γ -chain at methionine residues (Gross & Witkop, 1961) has met with considerable success in structural studies of rabbit IgG (Givol & Porter, 1965; Hill, Delaney, Lebovitz & Fellows, 1966) and a pathological human IgG (Press, Piggot & Porter, 1966*b*; Piggot & Press, 1967; Press & Piggot, 1967). This method of fragmentation of the immunoglobulin is valuable because of the low content of methionine in γ -chains, and the supposition that at least some of these residues would occupy constant positions. The latter supposition appears to be fulfilled at least in the Fc fragment of rabbit IgG (Givol & Porter, 1965; Hill *et al.* 1966). In the investigation of the *N*-terminal sequence of pooled rabbit IgG, however, Press, Givol, Piggot, Porter & Wilkinson (1966*a*) reported the identification of a methionine residue in position 35 that was present in only 30–35% of the molecules. The present study was undertaken to determine if the variability in this methionine position could be related to genetic polymorphism. In fact a methionine-for-threonine replacement in the inter-Fd–Fc area of the γ -chain was found to be allotypically related. The methionine residue in the *N*-terminal position 35 appeared

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as a fractional residue in all three allotypes, but the composition of the *N*-terminal peptides isolated differed among the allotypes.

MATERIALS

Rabbit IgG. Sera of rabbits known to be homozygous with respect to the heavy-chain allotype were generously supplied by Dr A. S. Kelus (University of Birmingham), with the exception of the single serum Aa2/Ab4,5, which was given by Dr J. J. Cebra (Johns Hopkins University). The IgG was isolated from the sera by Na_2SO_4 precipitation (Kekwick, 1940) followed by chromatography on DEAE-Sephadex A-50. The precipitate from 50 ml. of serum obtained with 18% (w/v) Na_2SO_4 was dissolved and reprecipitated at 14% (w/v) Na_2SO_4 . The second precipitate was dissolved in 20 ml. of 0.15M-NaCl and dialysed exhaustively against 0.02M-potassium phosphate buffer, pH 6.3, in the cold. The solution was clarified by centrifuging at 14000g for 30 min., and the supernatant was applied to a DEAE-Sephadex A-50 column (1.6 cm. $\times$ 25 cm.); after elution with 100 ml. of the same buffer, a linear gradient to 0.20M-potassium phosphate buffer, pH 6.3, was developed (600 ml. total volume). The extinction of the eluate was measured at 280 m μ ; appropriate fractions were pooled, concentrated by pressure dialysis against 0.15M-NaCl and freeze-dried. Each fraction was subjected to electrophoresis on cellulose acetate and to immunoelectrophoresis.

Fc fragment. Fc fragment was prepared from each allotypically homozygous rabbit IgG by papain digestion (Porter, 1959). A 100 mg. sample of IgG was dissolved in 7 ml. of 0.5M-tris-HCl buffer, pH 8.0, containing 2 mM-EDTA. To this was added 0.5 ml. of the same buffer containing dithiothreitol and papain such that the final solution was 5 mM with respect to dithiothreitol (150-fold molar excess of thiol relative to protein) and contained 1 mg. of papain (protein/enzyme weight ratio 100:1). After incubation at 37° for 1 hr., the digest was cooled to 0° in an ice bath and the free thiol groups were alkylated by the addition of buffered (0.1M-tris-HCl, pH 8.0) iodoacetic acid to a final concentration of 11 mM. The reaction mixture was kept for 1 hr. in the cold, then dialysed against 0.01M-tris-HCl, pH 8.0, for 48 hr. at 4° and finally against 5 mM-potassium phosphate buffer, pH 7.2, for a further 72 hr. The crystals were harvested by centrifuging at 14000g for 15 min., washed with 2 ml. of cold phosphate buffer twice and finally dissolved in 2 ml. of 0.1N-acetic acid. After filtration through a Millipore membrane, crystallization was repeated according to the above procedure.

Enzymes. Papain, trypsin and chymotrypsin were purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Chymotryptic activity of the trypsin was minimized by treatment with L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one, as described by Kostka & Carpenter (1964).

Dithiothreitol. Dithiothreitol (Cleland's reagent) was purchased from Calbiochem (Los Angeles, Calif., U.S.A.).

Iodo[1- ^{14}C]acetic acid and iodo[1- ^{14}C]acetamide. These were purchased from The Radiochemical Centre (Amersham, Bucks.).

METHODS

Reduction and isolation of chains. The IgG from rabbits homozygous for one allotype was dissolved in 0.5M-tris-

HCl, pH 8.0, containing 2 mM-EDTA, to a protein concentration of 20 mg./ml. Solid dithiothreitol was added to a final concentration of 10 mM (150-fold molar excess of thiol relative to protein) and the reduction mixture was incubated at 37° for 2 hr. At the end of this time the mixture was cooled to 0° and buffered (0.1M-tris-HCl, pH 8.0) iodoacetic acid added to a final concentration of 5.0 mM (this is only equivalent to 25% of the total added thiol). After 1 hr. the alkylation mixture was dialysed overnight in the cold against 10 mM-tris-HCl buffer, pH 8.0, to remove excess of reagent, and then exhaustively dialysed against *n*-propionic acid.

Separation of the chains of the partially reduced protein was accomplished by fractionation on Sephadex G-75 columns in *n*-propionic acid run at room temperature. The γ -chain fraction was concentrated by pressure dialysis against 0.1N-acetic acid, dialysed against the same solvent and freeze-dried. The light-chain fraction was exhaustively dialysed against 0.1N-acetic acid and freeze-dried.

Total reductions were carried out in 7M-guanidine hydrochloride in 0.5M-tris-HCl buffer, pH 8.2, at a dithiothreitol concentration of 50 mM, for 2 hr. at 37°. Alkylation with iodoacetic acid (0.11M) was performed at 0° for 1 hr.

Cyanogen bromide cleavage of the γ -chains. This was carried out in 70% (v/v) formic acid, as described by Givol & Porter (1965), but at 4° for 24 hr. rather than 25°. At the end of this time the reaction mixture was diluted tenfold with water and the reagents were removed by freeze-drying. Less than 5% of the original methionine remained after this treatment.

Other methods. Amino acid analysis of γ -chains and peptides was carried out as described by Prahl (1967). Before analysis for homoserine the hydrolysate was dissolved in 1 ml. of pyridine-acetate buffer, pH 6.5, and boiled for 1 hr. to convert homoserine lactone into homoserine (Ambler, 1965). The solvents and method of high-voltage paper electrophoresis were as described by Crumpton & Wilkinson (1965); mobilities of peptides are expressed relative to a lysine marker for cation movement and to a glutamic acid marker for anionic movement, the latter mobility being indicated as a negative quantity. Enzymic digests were performed in 0.1M- NH_4HCO_3 for 2 hr. at 37° at an enzyme concentration of about 0.20 mg./ μ mole of peptide. Subtractive Edman degradation of peptides was carried out by the method of Konigsberg & Hill (1962). Electrophoresis on cellulose acetate strips and in starch gel was performed as described by Press *et al.* (1966b), and immunoelectrophoresis as described by Scheidegger (1955). The ^{14}C -labelled compounds were counted in a Nuclear-Chicago liquid-scintillation counter, with the scintillation fluid described by Kinard (1957).

RESULTS

Isolation of heavy chain from allotypically homozygous rabbits

The final purification of the IgG from the sera of allotypically homozygous rabbits required chromatography on DEAE-Sephadex A-50 in phosphate buffer at pH 6.3. The behaviour of all three allotypes examined appeared similar under such conditions. About 20–25% of the IgG was unretarded in 20 mM-potassium phosphate buffer,

Table 1. *Recoveries of chains from mildly reduced and alkylated IgG*

The residual protein obtained from trough fractions is not shown, and hence the totals show less than 100% recovery.

γ -Chain allotype	γ -Chain		Light chain	
	E_{280} (%)	Weight (%)	E_{280} (%)	Weight (%)
Aa1	72	68	26	29
Aa2	61	63	27	26
Aa3	73	67	23	28

Table 2. *Amino acid compositions of heavy chains isolated from IgG from a single rabbit or a pool of rabbits homozygous for one allotype*

Amino acid compositions are reported as residues/mol. of mol.wt. 50 000 assuming a carbohydrate content of 6%.

Amino acid composition (residues/mol. of mol.wt. 50 000)

γ -Chain allotype ...	Aa1		Aa2		Aa3	
	Single	Pool	Single	Pool	Single	Pool
Ala	20.7	22.6	23.4	24.0	24.4	25.7
Arg	17.4	17.9	17.6	16.9	16.7	15.4
Asp	31.5	33.6	33.7	34.4	32.0	33.9
Cys·CH ₂ ·CO ₂ H	3.5	3.1	4.4	6.7	3.2	2.0
Cys*	8.8	9.5	7.3	5.8	10.5	10.7
Glu	36.7	33.9	37.0	36.0	37.9	38.5
Gly	32.9	33.4	32.7	33.1	33.6	37.0
His	6.2	6.4	6.4	6.5	6.6	6.0
Ile	16.0	15.8	14.7	14.3	14.5	14.0
Leu	32.2	29.3	32.1	30.6	33.2	29.7
Lys	22.3	24.6	23.4	24.5	24.5	23.1
Met†	5.3	4.7	4.9	4.4	6.3	5.9
Phe	14.5	13.8	13.7	13.4	15.2	14.3
Pro	39.9	39.0	38.3	35.7	39.2	35.7
Ser	45.8	44.2	46.0	45.6	42.2	45.9
Thr	47.5	48.1	48.6	45.1	42.0	48.9
Tyr	15.8	16.4	14.6	15.4	16.0	16.6
Val	38.9	40.4	40.0	39.2	38.9	40.3
Total	428	427	430	432	427	429

* Determined as cysteic acid (Moore, 1963).

† Average of methionine and methionine sulphone values (Moore, 1963).

but often it was contaminated by a small amount of pigmented protein, presumably haemoglobin. Of the retarded IgG, about 80% was eluted by 80–90 mM-buffer during the development of the linear gradient, and was free of contaminants as indicated by electrophoresis on cellulose acetate and immunoelectrophoresis. This fraction was used for the structural studies described below. The remaining IgG was eluted by the time the gradient had reached 0.12–0.14 M buffer, but was usually contaminated by β -globulins. The γ -chains were isolated from the IgG after partial reduction and alkylation by gel filtration in *N*-propionic acid

(Fleischman, Pain & Porter, 1962). The recoveries of γ -chains and light chains are shown in Table 1. Although Koshland, Englberger & Shapanka (1966) have reported poor recoveries of light chain from rabbit IgG unless total reduction was accomplished, our data suggested that such drastic measures were not necessary and were indeed undesirable, as in our hands such conditions appeared to lead to some alkylation of methionine residues. To avoid alkylation of other groups, only 0.25 mole of iodoacetate/mole of thiol group was added after mild reduction (cf. Crestfield, Moore & Stein, 1963). Electrophoresis in 8 M-urea-formate starch gel,

pH 3.7 (Edelman & Poulik, 1961), of a 2% (w/v) solution of γ -chain prepared as described here revealed only trace contamination with light chain.

Six preparations of γ -chain carrying a single allotypic specificity were isolated from rabbit IgG: two Aa1, one from a single rabbit of allotype Aa1/Ab5 and the other from a pool of five rabbits of allotype Aa1/Ab4; two Aa2, from single rabbits of allotypes Aa2/Ab4 and Aa2/Ab4,5; two Aa3, one from a single rabbit of allotype Aa3/Ab5 and the second from a pool of ten rabbits of allotype Aa3/Ab4. The amino acid analyses of γ -chains isolated from these preparations are given in Table 2. In view of the use of cyanogen bromide to cleave the γ -chain into smaller fragments for structural studies, the observation that the Aa3 allotype γ -chain contained approx. 1 mole of methionine more/50000 g. of protein than the γ -chains of the Aa1 and Aa2 allotypes was important. To locate this additional residue of methionine in the Aa3 γ -chain, Fc fragments were prepared from each allotypically homozygous IgG by papain digestion (Porter, 1959). The amino acid composition of each of these fragments is shown in Table 3. Despite the marked similarity, there did appear to be a variation in several residues. Significantly, however, the methionine content of all three was approximately the same, which is consistent with the sequence data of rabbit Fc fragment as reported by Hill *et al.* (1966).

Cyanogen bromide digest products

In view of the above finding, the additional methionine residue was sought outside the Fc fragment. γ -Chains carrying a single allotypic specificity were cleaved by cyanogen bromide, and the products were totally reduced and alkylated with iodo[1-¹⁴C]acetic acid (0.1 mc/m-mole) in 7M-guanidine hydrochloride. The fragments were then fractionated on a column of Sephadex G-100 in 6M-urea-0.2M-formate, pH 3.3, both the extinction of the eluate at 280 m μ and the radioactivity being measured. Elution patterns similar to the one shown in Fig. 1 were obtained for all three allotypes, in which the nomenclature employed is that used by Press *et al.* (1966a) and Cebra (1967). The relative positioning of these fragments is illustrated in Fig. 2. The smaller fragments (peptides C4a and C5), which should include the N-terminal fragment of peptide C1 (Press *et al.* 1966a), were pooled; the urea was removed by gel filtration on Sephadex G-25 in 0.05N-ammonia, the extinction of the eluate at 215 m μ being measured, and the peptide fraction was freeze-dried. When fractionated on a column (1.2 cm. $\times$ 280 cm.) of Sephadex G-50 in 0.05N-ammonia, the three peptides were found in the following elution positions (relative to the total column volume): for the N-terminal peptide, 53%, 53% and 51%; for peptide C4a, 68%, 69% and 68%; for peptide C5, 82%, 82% and 80%, for the

Table 3. *Amino acid compositions of Fc fragments prepared from IgG from rabbits allotypically homozygous at the a locus*

Amino acid compositions are expressed as residues/mol. of protein of mol.wt. 23000 (25000 mol.wt. of Fc monomer, containing about 7% of carbohydrate).

γ -Chain allotype ...	Amino acid composition (residues/mol. of mol.wt. 23000)			
	Pooled	Aa1	Aa2	Aa3
Ala	8.0	7.6	8.1	8.0
Arg	10.6	10.7	10.6	10.3
Asp	18.3	18.0	20.9	18.6
Cys $\cdot$ CH ₂ $\cdot$ CO ₂ H	0.7	0.8	0.8	0.8
Cys*	3.8	3.7	3.9	3.7
Glu	23.4	23.3	23.2	24.2
Gly	10.4	10.6	10.4	11.0
His	5.0	4.8	4.8	4.6
Ile	9.9	9.6	9.5	10.5
Leu	14.4	14.7	14.9	14.1
Lys	13.9	12.8	13.3	13.2
Met	3.6	3.7	3.8	3.9
Phe	7.9	7.5	7.7	7.8
Pro	20.3	20.4	20.6	21.4
Ser	18.9	19.0	18.7	19.2
Thr	14.8	15.8	14.6	15.9
Tyr	7.2	7.1	7.0	7.2
Val	16.8	16.4	16.4	17.4

* Determined as cysteic acid after performic oxidation (Moore, 1963).

Fig. 1. Fractionation of a cyanogen bromide digest of the γ -chain of the IgG from rabbits homozygous for the Aa3 allotype after total reduction and alkylation with iodo [1- 14 C]acetic acid. A 100 mg. sample of the digest was run on a column (120 cm. $\times$ 2.4 cm.) of Sephadex G-100 in 6 M-urea-0.2 M-sodium formate, pH 3.3. —, E_{280} ; ----, radioactivity.

Fig. 3. Fractionation of small cyanogen bromide peptides of the γ -chain of IgG allotype Aa1 (—) and Aa3 (----) on a column (1.2 cm. $\times$ 280 cm.) of Sephadex G-50 in 0.05 N-ammonia.

Fig. 2. γ -Chain of pooled rabbit IgG, illustrating the positions of methionine residues. Integral methionine residues are indicated by a full vertical bar and fractional or allotype-related methionine residues by a half bar. The numbers of residues between methionine residues are shown above the line. The positions of half-cystine residues that can be assigned are designated, as is the carbohydrate (CHO) moiety. The accepted nomenclature of the fragments is indicated below the chain.

allotypes Aa1, Aa2 and Aa3 respectively (Fig. 3). The composition of the *N*-terminal fragments was found to differ between the allotypes, whereas that of peptides C4a and C5 was the same for all three allotypes. These compositions are shown in Table 4. With the totally reduced and alkylated cyanogen bromide cleavage products of the Aa3 γ -chain, however, an additional peptide, peptide C1c, was found at 61% of the total column volume, the composition of which is also shown in Table 4. The absolute yields and yields relative to 100%

recovery of peptide C4a were calculated on the basis of the amino acid analyses and are shown in Table 5, which also includes the specific radioactivities of the peptides containing *S*-carboxymethylcysteine. Peptide C4a and the *N*-terminal peptide were radioactive, but peptide C1c was not, despite containing *S*-carboxymethylcysteine. Presumably the equivalent disulphide bond was split by gentle reduction during isolation of the chains and hence reacted with unlabelled iodoacetic acid.

The composition of peptide C1c was striking in its high content of proline, and was identical in composition with that peptide observed by Hill *et al.* (1966) to occupy the *N*-terminal position of the Fc fragment, named by them peptide VIIb. Sequential Edman degradation showed on analysis a fall in the molar content of a single amino acid at each step and hence revealed the *N*-terminal residues of peptide C1c to be *S*-carboxymethylcysteine (0.74-0.11; first step) followed by proline (7.34-6.29; second step). Gel filtration of a tryptic digest of peptide C1c on Sephadex G-25 in 0.02 N-ammonia revealed a peptide of the following composition (residues/mol.): aspartic acid, 1.00; threonine, 0.93; homoserine, 0.91; leucine, 1.02. This is in agreement with that of the peptide T25a isolated from the tryptic digest of peptide VIIb by Hill *et*

Table 4. *Amino acid compositions of several cyanogen bromide cleavage fragments from γ -chains from rabbits homozygous for one allotype*

Amino acid compositions are expressed as residues/mol. of peptide. The *N*-terminal peptide compositions are based on a homoserine content of 1 residue/mol. of peptide.

Peptide ...	Amino acid composition (residues/mol. of peptide)					
	<i>N</i> -Terminus of peptide C1* from			C4a* (VIIId†)	C5* (VII-1†)	C1c* (VIIb†)
	Aa1	Aa2	Aa3			
γ -Chain allotype ...						
Ala	0.85	1.43	1.58	—	1.04	—
Arg	0.93	0.81	0.18	2.01	1.00	—
Asp	1.14	2.53	1.86	—	1.06	1.16
Cys·CH ₂ ·CO ₂ H	0.80	0.67	0.65	0.66	—	0.78
Glu	2.84	3.95	4.60	1.79	1.97	1.08
Gly	3.94	5.05	5.44	1.44	1.11	2.65
His	0.13	0.29	0.15	—	2.67	—
Ile	0.50	0.79	0.28	—	0.98	0.97
Leu	3.60	4.06	4.01	2.01	1.05	3.00
Lys	0.21	1.80	1.02	—	0.99	2.03
Phe	0.66	1.62	1.74	—	—	1.84
Pro	1.74	1.59	1.59	2.44	0.91	7.34
Ser	4.00	5.36	5.17	3.48	2.72	1.03
Thr	4.18	4.55	3.01	0.99	1.00	1.04
Tyr	0.61	0.27	0.91	—	0.72	—
Val	2.38	2.53	1.74	1.00	—	1.11
Homoserine	1.00	1.00	1.00	1.01	—	1.00
Total	30-32	36-38	34-36	17	18	24

* Nomenclature of Press *et al.* (1966a) and Cebra (1967).

† Nomenclature of Hill *et al.* (1966).

Table 5. *Yields of small cyanogen bromide cleavage fragments obtained from γ -chains from rabbits homozygous for one allotype*

Shown are absolute yield and (in parentheses) relative yields based on 100% recovery of peptide C4a. The ¹⁴C content of the peptides containing *S*-carboxymethylcysteine, expressed in mc/ μ mole of peptide, is given in square brackets.

γ -Chain allotype	Peptide ...	Yield (%)			
		C5	C4a	C1c	<i>N</i> -Terminus of peptide C1
Aa1		46	65	0	37
		(71)	(100)	(0)	(57)
			[0.045]		[0.036]
Aa2		46	60	0	13
		(77)	(100)	(0)	(21)
			[0.034]		[0.052]
Aa3		38	50	42	23
		(75)	(100)	(80)	(45)
			[0.038]	[0.007]	[0.047]

al. (1966). A chymotryptic digest of peptide C1c was fractionated by high-voltage paper electrophoresis at pH 6.5 into three components, the compositions, mobilities and staining characteristics of which are shown in Table 6. Cebra, Steiner & Porter (1968b) have reported the sequence of the

C-terminal portion of the larger Fd-like fragment obtained by cyanogen bromide cleavage of the γ -chain of rabbit IgG, i.e. peptide C1. The compositions of the chymotryptic peptides shown in Table 6 are in excellent agreement with their reported sequences. Peptide C1c-2 is equivalent

Table 6. Amino acid compositions and other properties of chymotryptic peptides of peptide C1c

Peptide ...	Amino acid composition (residues/mol. of peptide)		
	C1c-3	C1c-2	C1c-1
Asp	1.00	—	—
CyS·CH·CO ₂ H	—	—	0.75
Glu	—	—	1.00
Gly	—	1.91	—
Ile	0.78	—	—
Leu	1.00	—	1.70
Lys	1.99	—	—
Phe	1.02	0.94	—
Pro	3.34	1.19	3.00
Ser	—	0.89	—
Thr	0.96	—	—
Val	—	1.00	—
Homoserine	1.03	—	—
Mobility*	+0.28	0	−0.85
Ninhydrin stain	Blue	Yellow-grey	Yellow

* Mobility during electrophoresis on paper relative to lysine (+1.0) or glutamic acid (−1.0).

Fig. 4. Fd-Fc sequence of the γ -chain of rabbit IgG (Cebra *et al.* 1968b) indicating the peptides isolated: (a) in the present work by the cyanogen bromide cleavage of the Aa3 γ -chain; (b) from the *N*-terminus of the Fc fragment obtained by cyanogen bromide cleavage (Hill *et al.* 1966); (c) by Cebra *et al.* (1968b) and in the present work from the tryptic peptides Ts2 isolated from fragment C1 and from γ -chains of various allotypes. The methionine-for-threonine replacement characteristic of Aa3 with respect to Aa1 and Aa2 allotypes is shown. The sequence shown is believed to be approximately residues 205–240, numbered from the *N*-terminal end of the γ -chain.

to their peptide C-Ts2-N, peptide C1c-1 to the *C*-terminal heptapeptide of their peptide C-Ts2-A1, and peptide C1c-3 to their peptide C-Ts2-B plus peptide T-T2-SA or to the identical peptide T25a of Hill *et al.* (1966) (see Fig. 4). Although the sequences of the chymotryptic and tryptic peptides of peptide C1c were not established, it appears conclusive that peptide C1c is indeed the equivalent of the *N*-terminal peptide of the Fc fragment; this

places the additional methionine residue of Aa3 γ -chain in the position preceding the *N*-terminal half-cystine residue of the Fc fragment, where threonine was placed by Cebra *et al.* (1968b). Peptide C1c had not previously been identified among the cyanogen bromide cleavage products of γ -chain prepared from pooled rabbit IgG (Cebra, Givol & Porter, 1968a). It has subsequently been isolated from two independent such preparations

(kindly provided by Dr J. M. Wilkinson) in 14% and 18% yields relative to the recovery of peptide C5.

Tryptic peptide containing the methionine-for-threonine replacement

Comparison of these results with those of Cebra *et al.* (1968b) suggested that the methionine residue was replacing a threonine residue. It was necessary to confirm this and to look for other allotype-related replacements in this section that might have been overlooked in the work with pooled IgG. As discussed above, analysis of the Fc fragments from the three allotypes (Table 3) had shown a very similar content of methionine, implying that the additional residue would not be found in this fragment when prepared from the Aa3 allotype IgG. An extension of the *N*-terminal sequence of Fc fragment was sought in the *N*-terminal peptide obtained from cyanogen bromide digestion of Fc fragment from Aa1 IgG, where alkylation of the papain digest with iodo[1-¹⁴C]acetic acid introduced ¹⁴C only into the half-cystine residue of the *N*-terminal fragment. The results of Hill *et al.* (1966) were confirmed, that the principal *N*-terminal sequence is Cys-Pro-Pro-, and that this peptide (i.e. peptide VIIb) did not contain a significant amount of additional *N*-terminal residues and so was not useful for such a purpose.

Cebra *et al.* (1968b) isolated a peptide, Ts2, from the tryptic digest of either fraction C1 or whole

heavy chain, that contained 31 amino acid residues and should include the methionine-for-threonine replacement and any other adjacent replacements that may occur. Therefore peptide Ts2 was isolated from a tryptic digest of the C1 fraction of Aa1 and of Aa2 IgG, and, to avoid the split of the additional methionine residue in Aa3 IgG, from the whole heavy chain by the method of Cebra *et al.* (1968b). A contaminating histidine-containing peptide (probably peptide T-T2-LB described by Cebra *et al.* 1968b) was removed by refractionation on a Sephadex G-50 column (1.9 cm. $\times$ 120 cm.) in 50 mM-ammonium hydrogen carbonate. Analysis and yield of peptide Ts2 from the different allotypes are given in Table 7. There was a reasonable agreement with the analysis reported for peptide Ts2 from the heavy chain of pooled IgG (Cebra *et al.* 1968b) except in peptide Ts2 from Aa3 allotype, which contained 0.7 mol. of methionine/mol. and a correspondingly lower content of threonine.

Chymotryptic digestion of peptide Ts2 from Aa1 and Aa2 allotypes gave the peptides reported by Cebra *et al.* (1968b), and good agreement with their results was found in the behaviour of the chymotryptic peptides on Sephadex columns, in their mobility on high-voltage electrophoresis on paper and in their amino acid composition (Table 8). Further Edman degradation of peptide C-Ts2-A1 (Table 9) gave the sequence Thr-CMCys-Ser-Lys-Pro-Thr-CMCys-(Pro₃Glu₂Leu₂) (where CMCys represents *S*-carboxymethylcysteine), in agreement

Table 7. *Amino acid composition of tryptic peptide Ts2 isolated from γ -chains allotypically homozygous at the a locus*

γ -Chain allotype ...	Amino acid composition (residues/mol. of peptide Ts2)		
	Aa1	Aa2	Aa3
Ala	1.02	1.21	0.93
Arg	—	—	—
Asp	—	—	—
Cys·CH ₂ ·CO ₂ H	1.84	2.12	2.05
Glu	1.02	1.18	1.10
Gly	1.84	2.16	2.11
His	—	—	—
Ile	0.78	0.96	0.86
Leu	1.85	2.01	2.21
Lys	2.86	3.04	2.70
Met	—	—	0.69
Phe	1.60	1.84	1.87
Pro	8.55	8.69	8.71
Ser	2.97	2.87	2.78
Thr	2.76	2.89	2.21
Tyr	—	—	—
Val	2.00	2.12	2.22
Total	31	31	31
Yield (%)	65	47	52

Table 8. *Amino acid composition and other properties of chymotryptic peptides of peptide Ts2*

		Amino acid composition (residues/mol. of peptide)				
γ -Chain allotype ...		Aa1 and Aa2				Aa3
Peptide		C-Ts2-A1	C-Ts2-C	C-Ts2-N	C-Ts2-B	C-Ts2-A1
Ala		—	1.00	—	—	—
CyS·CH ₂ ·CO ₂ H		1.83	—	—	—	1.68
Glu		1.13	—	—	—	1.01
Gly		—	—	1.67	—	—
Ile		—	—	—	0.89	—
Leu		2.00	—	0.19	—	2.00
Lys		0.92	—	—	2.18	0.87
Phe		—	—	0.91	0.92	—
Pro		3.96	0.96	0.93	3.00	4.02
Ser		0.89	0.87	0.97	—	0.91
Thr		2.01	—	—	—	1.24
Val		—	0.93	1.00	—	—
Homoserine		—	—	—	—	0.73
Elution volume on Sephadex G-50 in 0.05 N-NH ₃ (% of total column volume)		38–43	85–90	50–54	52–60	37–42
*Mobility						
pH 6.5		–0.40	0	0	+0.69	–0.24
pH 3.5		—	+0.14	+0.22	—	—
Ninhydrin stain		Light blue	Yellow–blue	Blue	Blue	

* Mobility during electrophoresis on paper relative to lysine (+1.0) or glutamic acid (–1.0).

Table 9. *Edman degradation of chymotryptic peptide C-Ts2-A1 from peptide Ts2 of Aa1 γ -chain*

n.d., Not determined. **Bold type** indicates the amino acid removed at each step.

		Amino acid composition (residues/mol. of peptide)							
Step no. ...		0	1	2	3	4	5	6	7
CyS·CH ₂ ·CO ₂ H		1.83	1.74	1.16	1.22	1.10	1.04	1.10	0.47
Glu		1.13	1.03	1.12	1.02	1.04	1.15	1.24	1.32
Leu		2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Lys		0.92	n.d.	n.d.	0.89	0.47	0.11	n.d.	n.d.
Pro		3.96	4.03	4.04	4.12	3.74	3.26	2.97	3.12
Ser		0.89	0.96	1.07	0.30	0.09	0.14	0.09	0.14
Thr		2.01	1.08	0.96	1.02	0.91	0.88	0.35	0.18
Recovery (%)			87	73	84	67	42	35	46

with the previous results. Hence it is concluded that the sequence of peptide Ts2 from allotypes Aa1 and Aa2 is identical with that reported for peptide Ts2 from the heavy chain of pooled IgG.

To help identify the peptides containing the methionine residue and decrease any risk of chymotryptic split at this point, peptide Ts2 from Aa3 allotype was alkylated at the methionine residue with iodo[1-¹⁴C]acetamide at pH 3.5 by the technique of Tang & Hartley (1967) before chymotryptic digestion. Peptide C-Ts2-A1, isolated as described

above, contained all the radioactivity and gave the analysis shown in Table 8; the alkylated methionine was converted into homoserine before hydrolysis for analysis (Tang & Hartley, 1967). The other chymotryptic peptides gave the same analysis as the equivalent peptides from Aa1 and Aa2 allotypes. Peptide C-Ts2-A1 could be fractionated further by electrophoresis at pH 6.5, and two peptides could be identified. One, staining yellow (mobility –0.4), had no radioactivity and gave the same analysis as peptide C-Ts2-A1 from Aa1 and

Aa2 allotypes, with no homoserine and 2 mol. of threonine/mol. of peptide. The other, radioactive, peptide (mobility -0.24) was present in much larger amounts and gave the same analysis except that it contained 0.8 mol. of homoserine and only 1 mol. of threonine/mol. of peptide. Confirmation of the position of the methionine-for-threonine replacement in this latter peptide was obtained by boiling it for 2 hr., whereupon the alkylated methionine was converted into homoserine with cleavage of the peptide at this point (Tang & Hartley, 1967). The resulting two peptides were separated by electrophoresis at pH 6.5 and on analysis gave the results expected from the sequence shown in Fig. 4. Hence it seems justifiable to conclude that in the mid-section of the heavy chain represented by the 31 residues of peptide Ts2 the methionine-for-threonine is the only allotype-related replacement.

DISCUSSION

Analysis of the γ -chains of rabbit IgG from the three major allotypes revealed an additional methionine residue in that of the Aa3. That this is indeed allotype-related rather than individual variation is based on the agreement between the results from both single rabbits and pools of serum obtained from five to ten rabbits of the same allotype at the α locus. Cyanogen bromide cleavage led to the isolation of the expected additional peptide and sequence studies of this and related peptides showed that a methionine-for-threonine replacement had occurred. The position of the replacement was found to be on the N -terminal side of the half-cystine residue that forms the N -terminal residue of the Fc fragment on papain hydrolysis. This position was also correlated with the more extensive sequence described by Cebra *et al.* (1968b) for this section of the γ -chain. Isolation and characterization of the relevant peptides from the γ -chains of the three allotypes showed that this is the only allotype-related replacement in this section of the γ -chain. As the relative recoveries of the peptides of interest were the same in all the allotypes (Table 7), it would appear that the sequences reported here are associated with the same class of rabbit IgG molecules. The analytical results (Tables 7 and 8) suggest that the replacement is only in about 70–80% of the Aa3 heavy chains, presumably reflecting a further heterogeneity in the genetic structure. Indeed, antigenic subdivisions of the main allotypes have been reported, and superimposed on this a small but appreciable proportion (10–20%) of IgG molecules seem to lack allotypic specificity of the α (or of the β) group (see Oudin, 1966).

There is no evidence that the methionine-for-

threonine replacement is reflected in a change of antigenic specificity, in contrast with the leucine-for-valine replacement at position 191 in human κ -chains, which results available at present suggest is the sole structural change on which the InV specificity depends (Milstein, 1966). The observations that the antigenic determinants of the α locus persist in the Fab fragment (Kelus *et al.* 1961; Marrack *et al.* 1962) although the area of the methionine-for-threonine replacement is degraded by papain digestion argue against its antigenic role.

The specific radioactivity of the S -carboxymethylcysteine of peptide C1c isolated from the γ -chain of the Aa3 allotype (Table 5) relative to that of the N -terminal peptide of peptide C1 and to that of peptide C4a indicates the disulphide bond formed by the half-cystine residue of peptide C1c is reduced and alkylated in the production of the γ -chain from intact IgG. This is in agreement with the observation of Cebra *et al.* (1968b), who found ^{14}C in peptide Ts2 prepared conversely from mildly reduced and radioactively alkylated pooled γ -chain. Mild reduction and radioactive alkylation of Fc fragment was found to label only the half-cystine residue in the N -terminal position, also in agreement with the observation of Cebra *et al.* (1968b) that the S -[^{14}C]carboxymethylcysteine of radioactively labelled γ -chain is found only in the C1 fragment on cyanogen bromide cleavage. Preliminary studies on the cyanogen bromide cleavage products of intact IgG from a rabbit homozygous for the Aa3 allotype indicate that at least in this particular allotype the half-cystine residue of peptide C1c forms a symmetrical inter- γ -chain bond (J. W. Prahl, unpublished work). It remains to be shown whether other inter- γ -chain disulphide bonds exist in the C1 section, and if allotypic variations in disulphide bonding occur.

Analysis of other purified cyanogen bromide-digest fragments (Table 4) suggests that allotype-related replacements also occur near the N -terminal end of the γ -chain; the low arginine content of the N -terminal peptide of Aa3 γ -chain compared with that of Aa1 and Aa2 and the low lysine content of the N -terminal peptide of Aa1 γ -chain compared with that of Aa2 and Aa3 are particularly striking. Detailed sequence studies of the N -terminal section of the γ -chain (J. M. Wilkinson, unpublished work) show that much more substantial differences occur in this area. This is in agreement with the immunological evidence that the antigenic differences are confined to the Fab fragments. The finding of apparently allotype-related sequence variation both in the N -terminal section and near the middle of the chain, some 200 residues apart, is surprising. If this is correct it suggests that these two sections of the chain may be related, perhaps in the steric structure. It could also be taken as strong evidence

that this γ -chain is coded by a single gene, contrary to several theories proposed to explain the origin of antibodies (see Lennox & Cohn, 1967; Cohen & Milstein, 1967).

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