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The Molecular Determinants of the A11 and A12 Allotypic Specificities in Rabbit Immunoglobulin*

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ABSTRACT: The finding that group a allotypes, segregating genetic markers determined by primary structure of the Fd region, are present on the distinctive heavy chains of several classes of rabbit immunoglobulin has posed a problem in understanding the nature of the genetic control of immunoglobulin synthesis. Recently a new system of allotypic markers, A11 and A12, detected by hemagglutination techniques has been identified on the γ heavy chain of rabbit immunoglobulin G. In the present paper it is shown that A11 correlated with methionine on the N-terminal side of the half-cystine participating in the inter-γ-chain bond, whereas A12 correlates with threonine at this position. The significance of the methionine–threonine interchange sharing the same chain with amino acid sequences determining the group a allotypes is considered in relationship to the genetic control of the biosynthesis of heavy and light chains of immunoglobulin.

Segregating genetic markers recognized by their differing intraspecies antigenic specificities were first reported by Oudin and named allotypes[1] (Oudin, 1956). These were shown to constitute two groups distinguished by their genetic behavior (Oudin, 1960). The group a determinants a1, a2, and a3 are present on the Fd portion of the heavy (H) chains and the group b determinants b4, b5, b6, and b9 (Dubiski and Muller, 1967) are present on the light (L) chain (Stemke, 1964). The determinants within each of groups a and b behave as alleles, and the two groups are not genetically linked to one another (Dubiski *et al.*, 1962).

Studies of peptides obtained by cyanogen bromide treatment indicated that the amino acid composition of a peptide present at the N-terminal end of the Fd fragment correlates with the group a allotype of the γ chain (Koshland, 1967; Porter, 1967; Koshland *et al.*, 1968; Prahl and Porter, 1968). Recently Wilkinson (1969a) has related these differences directly to amino acid sequence variations for the a1 and a3 specificities. A replacement of methionine for threonine in the hinge region of rabbit γ chain which appeared to correlate with allotype in pools of a3 and of a1 IgG has been reported (Porter, 1967; Prahl and Porter, 1968). Methionine at this position was found in 60–80% of the molecules of the a3 pool. Koshland (1967) was unable to confirm this replacement in purified antiphenylarsonic acid and antiphenyl β-lactoside antibodies from rabbits homozygous for the a3 determinant.

Recently Mandy and Todd (1968, 1969, 1970) have described another pair of allotypic specificities, A11 and A12. These specificities are detected by inhibition of hemagglutination. Their determinants are in the hinge region of the γ chain. Each of these specificities is associated with each of the group a allotypes so that all combinations are possible, *e.g.*, a1,A11, a1,A12, etc. Despite this variation, breeding studies have shown them to be linked to the group a specificities (Zullo *et al.*, 1968; Mandy and Todd, 1970).

We report here an investigation of the interchange of methionine and threonine in γ chains of IgG from rabbits homozygous for the group a allotype determinants as well as for A11 and A12. It will be shown that the presence of methionine correlates with the presence of the A11 and the presence of threonine correlates with A12, while the composition of the N-terminal peptide (Koshland, 1967; Prahl and Porter, 1968; Wilkinson, 1969b) remains characteristic of the group a allotype.

Materials and Methods

Serologic Methods. Typing for groups a and b was done by interfacial precipitation reactions (ring tests) using antiallotype sera prepared by injection of rabbits with rabbit antiovalbumin specific precipitate following the method of Oudin (1960). Typing for A11 and A12 was done by inhibition of hemagglutination as described by Mandy and Todd (1969, 1970).

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[1] The nomenclature employed for rabbit immunoglobulins, their chains, and fragments follows that recommended by a Committee of the World Health Organization (1964). The notation for allotypes is that of Dray *et al.* (1962) except that the initial A has been omitted for those specificities assigned to a definite genetic group, *e.g.*, a1 in lieu of Aa1.

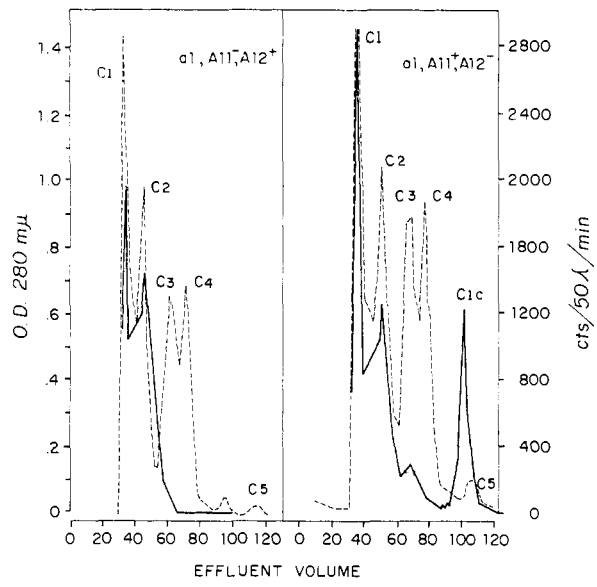

FIGURE 1: Gel filtration of cyanogen bromide reaction product through a 100 × 2.5 cm column of Sephadex G-100 in pH 3.2 0.05 M formate buffer, 6 M with urea. (·····) OD_{280} and (——) radioactivity. Numbers over the peaks indicate the peptides as shown in Figure 2.

Reduction and Isolation of Chains. IgG was obtained from the sera of individual rabbits by precipitation with 18% Na_2SO_4 (Kekwick, 1940) or 1.75 M $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose (Levy and Sober, 1960). IgG from rabbits homozygous for group a and A11 or A12 determinants was dissolved at a concentration of 10 mg/ml in 1.0 M Tris-HCl buffer (pH 8.2) containing EDTA (2mM). After deaeration with a stream of nitrogen, solid dithiothreitol (Calbiochem) was added to a concentration of 20 mM. The mixture was incubated for 2 hr at 37° and then cooled to 0°. A solution of [1-^{14}C]iodoacetic acid (0.6 mCi/mmole; ICN Corp.) in 0.1 M Tris-HCl (pH 8.2) buffer was added to a final concentration of 10 mM. After 1 hr the mixture was dialyzed in the cold against 10 mM Tris-HCl buffer (pH 8.0) and then against 1 N propionic acid. The chains were separated by gel filtration using Sephadex G-100 in 1 N propionic acid at room temperature (Fleischman *et al.*, 1962). The fractions containing the γ chains were pooled, dialyzed against 0.1 M acetic acid, concentrated by pervaporation, and lyophilized.

Cyanogen Bromide Cleavage. The γ chains were dissolved in 70% formic acid at a concentration of 10–15 mg/ml. Solid cyanogen bromide (Eastman Kodak) was added (10 mg/mg of γ chain) (Givol and Porter, 1965). After the reaction mixture had stood at room temperature in the dark for 24 hr, it was diluted tenfold with distilled water, and the solution was lyophilized. The product was dissolved in 0.05 M sodium formate buffer (pH 3.2) 6 M with urea and filtered through a column (100 × 2.5 cm) of Sephadex G-100 in the same buffer.

Fractions from peak C5 and its associated radioactive peak C1c (Figure 1) from the A11 IgG were pooled, freed of urea by gel filtration through a column (40 × 2.5 cm) of Sephadex G-25 in 0.05 M NH_4OH, and lyophilized.

Isolation of N-Terminal Peptides of C1 and C2 Fractions. The N-terminal peptides were isolated from the C1 and C2 fractions as described by Press *et al.* (1966) and Wilkinson (1969a). After dialysis against 0.5 N acetic acid, the pools of C1 and C2 fractions from each allotype were concentrated by lyophilization, totally reduced and alkylated in 7 M guanidine hydrochloride, and refractionated by gel filtration on a Sephadex G-50 column (180 × 1.9 cm) in 0.05 N NH_4OH. The N-terminal peptide was eluted at 49–52% of the column volume.

Amino Acid Analyses. Amino acid analyses were performed as described previously (Prahl, 1967) except that a Spinco Model 120C amino acid analyzer was used with 55 cm and 8 cm columns for the separation of acid and neutral plus basic amino acids, respectively, for all analyses. For peptides a recorder range expansion circuit was employed.

Isolation of the Ts2 Peptide. This was accomplished by a modification of the method described by Cebra *et al.* (1968). The mildly reduced and radioalkylated γ chain at a concentration of 20 mg/ml was digested with trypsin (L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated, Worthington) at a concentration of 0.2 mg/ml for 4 hr at pH 8.0 and 37° in a Radiometer autotitrator. At the end of this time the pH was brought to 4.0 with glacial acetic acid, and the digest was kept in the cold overnight. The resultant precipitate was removed by centrifugation, and the supernatant was fractionated on a column of Sephadex G-50 (180 × 1.9 cm) in 0.05 M NH_4OH. The radioactive fraction, which eluted at 38–42% of the total column volume, was subjected to electrophoresis at pH 3.5. The radioactive peptides were identified by radioautography. The region containing the major peptide (mobility of 0.20 relative to a lysine marker) was sewn to a second sheet of paper and reelectrophoresed at pH 6.5. The Ts2 peptide, neutral at this pH, was eluted and analyzed.

Chymotryptic Peptides of the Ts2 Peptide. The Ts2 peptides were digested with α-chymotrypsin (Worthington) in 0.1 M NH_4HCO_3 for 2 hr at 30° at an enzyme concentration of approximately 0.2 mg/μmole of peptide. The digest was then applied to paper and electrophoresed at pH 6.5. Peptide C-Ts2-B (see Figure 4) moved with a mobility of +0.70 relative to lysine (+1.0) and C-Ts2-A1 with a mobility of −0.37 relative to aspartic acid (−1.0). Peptides C-Ts2-C and C-Ts2-N, present in the neutral band, were resolved by descending chromatography in 1-butanol–acetic acid–water (180:53:266, v/v).

Results

Five preparations of IgG were obtained from individual rabbits possessing only one each of the group a and the A11 or A12 determinants. Of the six possible permutations, only the a2,A11 was not available for this study. The amino acid compositions of the γ chains obtained from each of them are given in Table I. The same general variations in composition between allotypes were observed as have been reported by Koshland *et al.* (1968). Most significantly those γ chains isolated from IgG bearing the A11 determinant were seen to contain approximately 1 mole of methionine more per mole of γ chain than those isolated from IgG bearing the A12 determinant.

The elution patterns obtained by filtration through Sephadex G-100 of the fragments from cyanogen bromide treatment of the a1,A11 and the a1,A12 γ chains are shown in Figure 1. Those from a2,A12 and a3,A12 were similar to that of a1,A12, and that from a3,A11 was similar to that of a1,A11. The fractions are identified using the symbols previously employed (Press *et al.*, 1966; Cebra, 1967; Prahl and Porter, 1968). The

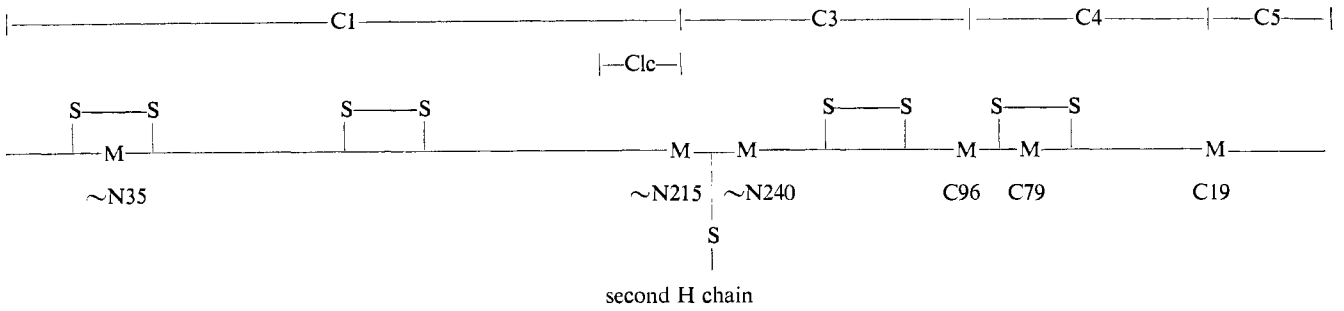

FIGURE 2: Structure of H chain of IgG. M indicates position of methionines.

TABLE I: Amino Acid Composition of H chains.[a]

	H-Chain Allotype				
	a1, A12	a1, A11	a2, A12	a3, A12	a3, A11
Ala	22.2	21.6	23.2	24.7	24.2
Arg	17.0	16.6	16.2	15.4	15.4
Asp	32.0	32.9	32.7	32.4	32.1
$CysCH_2CO_2H$	4.2	4.1	3.7	3.0	3.3
Cys	8.8	9.1	10.2	10.3	9.9
Glu	36.1	37.1	37.4	38.0	38.5
Gly	31.0	32.5	31.4	33.9	32.9
His	6.1	6.1	5.9	6.6	6.1
Ile	14.9	15.7	13.5	14.3	13.9
Leu	30.2	29.3	31.2	31.6	30.7
Lys	23.5	22.0	22.7	22.8	22.7
Met	4.5	5.6	4.6	4.7	5.6
Phe	13.8	13.6	13.3	14.5	14.2
Pro	39.0	39.0	40.0	38.5	37.4
Ser	53.0	49.5	52.1	50.5	50.4
Thr	50.3	50.1	50.0	45.8	47.0
Tyr	14.2	14.9	13.9	15.6	17.0
Val	39.5	40.8	38.1	38.4	38.7
Total	440.3	440.5	440.1	441	440.3

[a] Compositions are reported as amino acid residues per 440 residues. Cysteine and methione were determined as cysteic acid and methionine sulfone, respectively (Moore, 1963).

origin of the peptides in this fraction is indicated in Figure 2. Fractions C1 and C2 are the polymeric and monomeric forms of the N-terminal half of the γ chain. Since it had been found previously that the N-terminal half-cystine of the C1c peptide was reduced under the mild conditions employed for isolation of the chains (Cebra *et al.*, 1968; Prahl and Porter, 1968), the presence of a radioactive peak emerging with the late fractions from A11 γ chain was suggestive of the presence of the C1c peptide. That this was indeed the case was verified by pooling the fractions after the C4 peak, desalting the pool by filtration through Sephadex G-25, lyophilizing, and refractionating each pool using a column (180 × 1.9 cm) of Sephadex G-50 in 0.05 N NH_4OH. Radioactivity and OD_{215} were determined on the eluate fractions. The results obtained with the fractions from a1,A11 and a1,A12 γ chains are presented in Figure 3. Again the a2,A12 and the a3,A12 fractions gave elution profiles similar to that of a1,A12, whereas that from a3,A11 was similar to that from a1,A11. The C-terminal octadecapeptide C5 from all the allotypic variants was eluted between 80 and 84% of the column volume. In all cases it possessed the same amino acid composition as has been previously reported for this peptide (Givol and Porter, 1965). A radioactive peptide was eluted at 57–60% of the column volume from those fragments from γ chain bearing the A11 determinant. This peptide was shown to be the C1c peptide by amino acid analysis (Table II). No corresponding peptide was eluted from the fragments from γ chain bearing the A12 determinant. Based on an assumed recovery of 100% for the C5 peptide, the recovery of the C1c peptide was 93% from a1,A11 γ chain and 87% from a3,A11 γ chain.

The sequence adjacent to that of the methionine replacement was again investigated as had been done in the previous

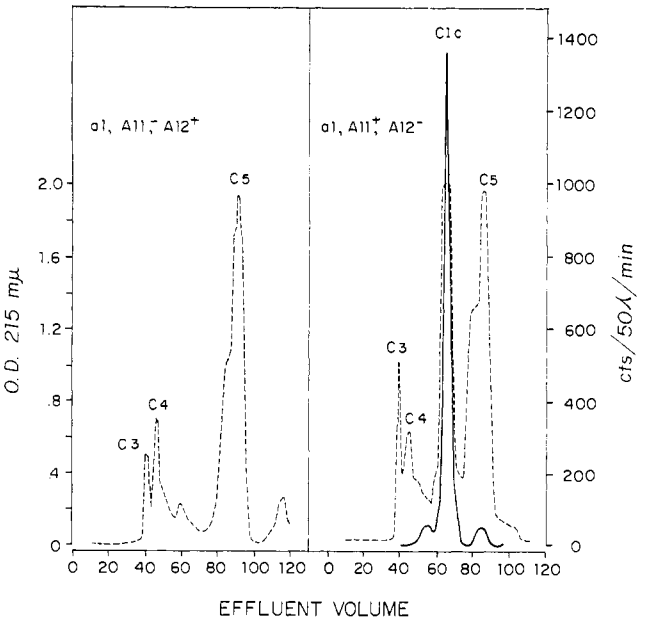

FIGURE 3: Gel filtration of C5 and associated radioactive peak through a 180 × 1.9 cm column of Sephadex G-50 in 0.05 M ammonium hydroxide. (·····) OD_{215} and (——) radioactivity. Numbers over the peaks indicate the peptides as shown in Figure 2.

study (Prahl and Porter, 1968) by the isolation of the Ts2 peptide from the tryptic digests of the γ chains of the various allotypes (Cebra *et al.*, 1968). The amino acid compositions of these peptides are given in Table III. As anticipated the Ts2 peptides from the A11 allotype γ chains contained approximately 1 mole of methionine/mole of peptide. This was accompanied by a decrease of approximately 1 mole of threonine/mole of peptide from that seen in the Ts2 peptides isolated from the A12 γ chains. The peptides obtained by chymotryptic digestion of Ts2 were isolated and on analysis gave amino acid compositions consistent with the sequence previously established as shown in Figure 4. There was no evidence in this study of the partial methionine–threonine interchange previously seen (Prahl and Porter, 1968).

TABLE II: Amino Acid Composition of C1c Peptides.[a]

	Allotype of H Chain	
	a1, A11	a3, A11
Asp	1.02	0.96
$CysCH_2CO_2H$	0.76	0.62
Glu	1.15	1.05
Gly	2.09	2.08
Ile	0.95	0.82
Leu	2.90	2.92
Lys	1.91	1.99
Phe	1.87	1.71
Pro	6.72	7.09
Ser	0.89	1.09
Thr	0.98	0.92
Val	1.09	0.95
Homoserine	0.96	0.94
Recovery (%)	93	87

[a] Compositions are reported as amino acid residues per mole of peptide. Recoveries are based on relative yield of C5 peptide.

TABLE III: Amino Acid Composition of Ts2 Peptides.[a]

	a1, A12	a1, A11	a3, A12	a3, A11
Ala	1.01	0.97	0.97	1.05
$CysCH_2CO_2H$	1.74	1.65	1.83	1.77
Glu	1.00	1.03	0.97	1.07
Gly	2.11	2.01	2.10	2.05
Ile	0.97	0.89	0.93	0.88
Leu	1.93	2.00	2.03	2.07
Lys	2.83	2.97	2.76	2.87
Met		0.93		0.86
Phe	1.84	1.91	2.01	1.89
Pro	8.87	8.73	8.91	9.02
Ser	2.78	2.84	2.79	2.83
Thr	2.92	2.02	3.03	2.09
Val	2.03	1.97	1.90	1.97

[a] Compositions are reported as amino acid residues per mole of peptide.

The N-terminal fragments obtainable from a portion of the C1 and C2 peptides have been shown to possess compositions characteristic of their group a allotype (Koshland, 1967; Prahl and Porter, 1968; Wilkinson, 1969a). The amino acid compositions and yields of these peptides as obtained in the present study are presented in Table IV. The compositions for a1 and a3 were found to be independent of the nature of the A11 and A12 allotypes and agreed with those previously reported.

Discussion

From the results presented above it is concluded that the methionine–threonine interchange in the hinge region of rabbit γ chain is related to the A11 and A12 determinants, being methionine in the case of those γ chains bearing the A11 determinant and threonine in those bearing the A12 determinant. In addition the compositions and by inference the primary sequences which characterize the N-terminal peptides of the group a allotypes a1 and a3 (Wilkinson, 1969a) are unaffected by a change in the A11 and A12 determinants.

In contrast to the allotypic specificities of groups a and b, which are normally identified by precipitation reactions, the allotypic specificities A11 and A12 are detected by hemagglutination (Mandy and Todd, 1968, 1969, 1970). The studies have shown that the A11 and A12 determinants are on the $(Fab')_2$ fragment obtained by pepsin digestion of IgG but are not on the Fab or Fc fragments obtained by digestion with papain. The integrity of the inter-γ-chain disulfide bond was found to be necessary for the detection of the specificity in IgG. The specificity was lost on mild reduction by mercaptoethanol but

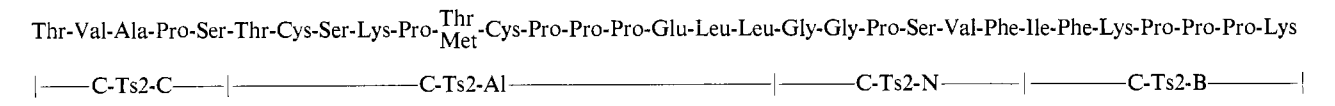

FIGURE 4: The Ts2 peptide of rabbit γ chain (Cebra *et al.*, 1968; Prahl and Porter, 1968). The chymotryptic peptides isolated are indicated, and the methionine–threonine interchange characteristic of the A11, A12 allotypes is shown. This sequence is approximately residues 205–236 numbered from the N-terminal end of the γ chain and encompasses the hinge region.

TABLE IV: Amino Acid Compositions of N-Terminal Peptides Obtained from C1 and C2 Fractions.[a]

	a1, A12	a1, A11	a2, A12	a3, A12	a3, A11
Ala	0.97	0.97	2.03	1.59	2.03
Arg	1.09	1.12	1.41	0.21	0.33
Asp	1.18	1.34	2.86	1.64	1.56
$CysCH_2CO_2H$	0.81	0.83	0.90	0.77	0.76
Glu	2.98	3.25	3.90	4.05	3.93
Gly	4.91	4.80	4.76	4.05	5.57
His	0.14	0.17	0.24	0.18	0.17
Ile	0.50	0.60	1.03	0.27	0.47
Leu	4.17	4.31	3.86	3.55	3.60
Lys	0.36	0.38	1.97	0.91	0.97
Phe	0.85	0.79	1.48	1.05	1.43
Pro	2.37	2.27	2.90	1.18	1.67
Ser	4.96	4.75	5.17	4.64	5.10
Thr	5.05	5.05	5.55	2.73	3.70
Tyr	1.00	0.98	1.17	1.18	1.47
Val	2.70	2.58	2.79	1.59	1.73
Homoserine	1.05	1.05	1.00	0.95	0.98
Yield (%)	42	34	22	21	19

[a] Compositions are reported as amino acid residues per mole of peptide.

returned on reoxidation. These experiments indicated that at least an important part of the determinant must lie in the hinge region of the γ chain. The present study supports this supposition.

Earlier structural studies of rabbit γ chain had also placed a methionine–threonine interchange in the hinge region (Prahl and Porter, 1968). Since methionine was found only in a3 molecules in these earlier studies, the presence of methionine in this region was interpreted as being related to this allotype. Samples of the IgG preparations used in this study, isolated from pools of a1 and a3 sera, were provided by R. R. Porter. Typing revealed the A11 specificity to be present on a3 but absent from the a1 IgG. At this time the system for determining A12 (Mandy and Todd, 1970) was not yet available. Subsequently sera from the a3 rabbits used by M. E. Koshland were supplied by her for typing. At this time the system for determining A12 was available. These sera were found to lack the A11 and possess the A12 specificities. It is significant that she had been unable to detect methionine in the hinge region of specific antibodies prepared from these sera. These findings taken in conjunction with the results reported here support the conclusion that the methionine–threonine interchange is related to the A11, A12 group of allotypic specificities rather than those of the group a. Methionine would relate to the A11 determinant, and threonine to its counterpart, A12. The lower recoveries of the C1c peptide seen in some of the a3 IgG pools in the previous work as compared with those found here may have resulted from heterozygosity with respect to the A11, A12 determinants. These studies point out once again the problem of selection in genetic studies on restricted populations, as it is now apparent that the inability to detect the methionine in the a1 IgG used in the earlier study was due to the unrecognized homozygosity of the A12 determinant.

The N-terminal peptides isolated from the fragments from digestion with cyanogen bromide have been shown to have compositions characteristic of each of the group a determinants (Koshland, 1967; Prahl and Porter, 1968). Wilkinson (1969a) has extended these observations by identifying significantly different primary sequences for the a1 and a3 peptides. The results presented here show that the composition of the N-terminal peptides of the a1 and a3 alleles remain characteristic of the group a allotype, quite independent of the nature of the A11 or A12 determinant.

The observations presented here require reconsideration of the suggestion (Prahl and Porter, 1968) that the finding of sequence variations related to a single allotype in both the N-terminal section and the hinge region would indicate a single gene to code for the entire γ chain. It now seems clear that γ chains exist which are a1,A11; a1,A12; a2,A11; a2,A12; a3,A11; and a3,A12. Two mechanisms of genetic control

might be visualized to explain how these chains arose. In the first possibility, synthesis of the γ chains would be governed by an interaction of a gene controlling the group a allotypic determinant with another gene at a separate but closely linked locus which controls the synthesis of that portion of the γ chain carrying the A11 and A12 determinants. If this be true, one could also visualize interaction of the genes controlling the group a determinants with other genes at still other loci for each of the Fcα (Kindt *et al.*, 1968; Wilkinson, 1969b), Fcμ (Todd and Inman, 1967), and Fcϵ (Kindt and Todd, 1969) determinants.

In the second possibility, separate but closely linked loci for γ, α, μ, and ϵ genes would be assumed. The α, μ, and ϵ loci would each have three alleles carrying information for the a1, a2, or a3 determinants. In this mechanism the γ locus would seem to require a minimum of six possible alleles, one for each of the six γ chains detailed above. If this be true, how did these distinct, but interrelated genes arise in a genetic region where, for man at least, recombination appears to be rare?

A similar dilemma exists in the case of the immunoglobulin L chains of man. Arguments for the two-gene concept have been advanced in this case (Dreyer and Bennett, 1965; Hood *et al.*, 1966a,b, 1967; Hood and Ein, 1968). The possibility of interaction of maternal and paternal genetic information in the synthesis of a single γ chain is being examined (Prahl *et al.*, 1969).

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