

GENETIC VARIANTS OF γ G4 GLOBULIN

A UNIQUE RELATIONSHIP TO OTHER CLASSES OF γ G GLOBULIN*

BY H. G. KUNKEL, M.D., F. G. JOSLIN, G. M. PENN, M.D., AND J. B. NATVIG, M.D.

(From *The Rockefeller University, New York 10021, and the Research Institute of Rheumatology, Oslo, Norway*)

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The γ G4 class of γ -globulin has aroused special interest because it differs most strikingly from the other γ G subgroups in a variety of properties; these include the rapid electrophoretic mobility of both the whole protein and the Fc fragment (1), the lack of complement-fixing ability (2), and multiple antigenic differences (3, 1, 4). Recent chemical studies have demonstrated at least 14 amino acid differences for the Fc fragment of one γ G4 protein as compared with the major γ G1 type (5). It is not clear at present what these differences relate to, since different genetic variants within a subgroup characteristically show multiple differences. This is particularly true for the γ G3 type where the Gm(b) type heavy chain differs from the Gm(g) type in at least five different antigens on the Fc fragment (6, 7). No genetic variants of the γ G4 proteins have been described thus far.

The present studies were undertaken after a number of antigenic differences were noted among γ G4 myeloma proteins. These were localized to the Fc fragments and the possibility that they represented genetic markers was investigated. Evidence obtained from studies on normal sera showed that two genetic variants were involved. The surprising finding was made that the differences noted among γ G4 myeloma proteins also related to antigens shared with other γ G classes.

Materials and Methods

Sera and Proteins.—Myeloma proteins were isolated from sera of patients with multiple myeloma by zone electrophoresis or by a combination of zone electrophoresis followed by Sephadex chromatography (1). Normal caucasian sera were obtained from blood donors at the New York Blood Center through the kindness of Dr. F. H. Allen. A γ G4 heavy chain disease serum was obtained from Dr. K. Bloch and the protein isolated by zone electrophoresis.

Enzymatic Digestion.—Digestion with papain was performed at an enzyme to protein ratio of 1:100 in 0.002M EDTA and 0.01M cysteine at pH 7.5 and 37°C. It was carried out for 2 hr for the isolated myeloma proteins as well as for the isolated γ -globulin from normal serum.

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The Fc fractions were separated by zone electrophoresis in pevikon by procedures published previously (1).

Detection of γ G1-3-4 and γ G2-4 Antigens.—Hemagglutination inhibition techniques were utilized as described previously (4). Two antisera were utilized primarily for the γ G-1-3-4 antigen. The first was made in a rabbit against the γ G1 protein Gr and the second was made in a cynomolgus monkey against the γ G4 protein Ge. Both antisera were absorbed with pepsin Fr II and two γ G2 myeloma proteins. As the protein coat, either anti-Rh antibodies or myeloma proteins coupled to red cells by BDB were utilized (4).

For the γ G2-4 antigen two antisera were utilized. Both were raised in cynomolgus monkeys; one was against the γ G2 protein Ne and the other against the γ G2 protein He. Only red cells coated with myeloma proteins were utilized in this system; various γ G2 myeloma proteins were attached to red cells with BDB and either of the γ G4 myeloma proteins He or Ma was used similarly.

Genetic Typing.—This was carried out for Gm(a) (z), (b), (g), and (n) by procedures described previously (8). The “non a” and “non g” antigens were similarly detected as described in reference (4). Heteroantisera from rabbits and cynomolgus monkeys were utilized in all instances, and anti-Rh coats were employed for each system except Gm(n).

RESULTS

The γ G1- γ G3- γ G4 Antigen.—Through the use of hemagglutination-inhibition techniques it has been possible to define a wide variety of antigens occurring in different parts of the molecules of various γ G globulins. Some of these represent subgroup specific antigens (1), others represent genetic markers (6, 7), and still others are antigens shared by certain subgroups but absent in others (4). Among the latter type for the Fc fragments are the following: γ G2- γ G3, γ G1- γ G2- γ G3, γ G2- γ G3- γ G1 Gm(f), and γ G2- γ G3 Gm(b). The last named, for example, has been termed “non g” since it occurs in all γ G2 proteins and only in the Gm(b) type of γ G3 proteins. It is absent in all γ G1 and γ G4 proteins and in the Gm(g) variant of γ G3.

Another shared antigen similar to those described above is the γ G1- γ G3- γ G4 antigen which was at first thought not to be unusual except that all γ G2 proteins were completely negative. However, more extensive study of γ G4 proteins indicated that not all contained this antigen. Table I indicates the results of one system used for detection of the γ G1- γ G3- γ G4 antigen. An anti-serum against a γ G4 protein absorbed with two γ G2 proteins plus pepsin-treated FrII was used as the agglutinator against anti-D coated red cells. Proteins of the γ G1 and γ G3 subgroups inhibited to low concentrations while γ G2 proteins failed to inhibit. Most γ G4 proteins inhibited in a fashion similar to the γ G1 and γ G3 types. However, definite exceptions were found and proteins He and Mo, shown in Table I, completely failed to inhibit. A total of 27 γ G1 proteins of both Gm(f) and Gm(az) types all reacted similarly. Eleven γ G3 proteins of Gm(b) and Gm(g) types also inhibited similarly. All of 11 γ G2 proteins (Gm(n) and Gm(n-) types) were completely noninhibitory. Some of these other proteins are listed in Table II. 23 γ G4 proteins were studied with 19 positive and 4 negative, thus giving an incidence of 17% for the negative

type. Exactly parallel results were obtained when a rabbit antiserum to a γ G3 protein was used as the agglutinator. The antigen was localized to the Fc fragment of the γ G4 protein as well as to those of the other subgroups. One γ G4 heavy chain disease protein contained the antigen. No reaction was obtained with γ M, γ A, or γ D proteins.

Special analyses were carried out on the four γ G4 proteins that failed to react. They were clearly γ G4 proteins by a wide variety of criteria. Two different γ G4 antisera typed them as γ G4 proteins both by precipitation and hemag-

TABLE I
Comparison between Proteins of Different Subgroups with Respect to Inhibition of Agglutination in the γ G1-3-4 System

Inhibitor	Inhibitor protein concentration, mg/cc				
	0.12	0.03	0.008	0.002	0.0005
Tr (γ G1, κ)	0	0	0	tr	2
De (γ G1, λ)	0	0	0	1	2
Sm (γ G2, κ)	2	2	2	2	2
Ca (γ G2, λ)	2	2	2	2	2
Vi (γ G3, λ)	0	0	tr	tr	2
Jo (γ G3, κ)	0	0	0	1	2
St (γ G4, λ)	0	0	0	0	2
No (γ G4, κ)	0	0	0	tr	2
Da (γ G4, κ)	0	0	tr	1	2
La (γ G4, κ)	0	0	0	1	2
He (γ G4, κ)	2	2	2	2	2
Mo (γ G4, κ)	2	2	2	2	2

Coat-anti D He.

Agglutinator-monkey anti- γ G4 Ge absorbed with pepsin FrII and γ G2 proteins Sm and Ne.

glutination. They lacked the γ G1-2-3 antigen, as do other γ G4 proteins. They lacked the γ G2-3 antigen found in all γ G2 proteins. Each of the isolated proteins were subjected to papain digestion and the unique fast Fc component characteristic of γ G4 proteins was always obtained. The proteins lacked all the known Gm genetic markers (the eight major types were screened); in addition, these proteins lacked non a and non g. Two of the proteins were aggregated with BDB along with controls from the other subgroups. Only the γ G4 proteins, including the two under special study, failed to fix complement.¹

The γ G2- γ G4 Antigen.—Since the four γ G4 proteins that lacked the γ G1-3-4

¹ The authors are indebted to Dr. Vincent Agnello for these determinations.

antigen appeared similar to γ G2 proteins in this respect, they were studied for other similarities. A monkey antiserum to the γ G2 protein Ne was found to react with these four γ G4 proteins after absorption with a γ G1, γ G3, and γ G4 protein containing the γ G1-3-4 antigen. Table III shows some of the results

TABLE II
Myeloma Proteins of Different γ G Classes and Genetic Types

Protein	Subgroup and genetic type	γ G1-3-4 antigen*	G2-4 antigen*
Tr	γ G1 (f)	++	0
De	" (az)	++	0
Gi	" (az)	++	0
Sn	" (f)	++	-
Ja	" (f)	++	0
Ti	" (az)	++	0
Ba	" (f)	++	0
La	" (az)	++	0
Vi	γ G3 (g)	++	0
Jo	" (b)	++	0
Jn	" (b)	++	0
Br	" (b)	++	0
Sm	γ G2 (n)	0	++
Ca	" (non g)	0	++
Ne	" (n)	0	++
Sp	" (non g)	0	++
Th	" (non g)	0	++
St	γ G4	++	0
No	"	++	0
Da	"	++	0
La	"	++	0
Ro	"	++	0
Ke	"	++	0
Le	"	++	0
He	"	0	++
Ma	"	0	++
Mo	"	0	++

The presence of the γ G1-3-4 and γ G2-4 antigens are indicated.

* From hemagglutination inhibition; ++, inhibition at <0.008 mg/cc; 0 = no inhibition at >0.12 mg/cc.

with this system employing hemagglutination inhibition techniques analogous to those for the γ G1-3-4 antigen. Exactly the reverse relationship of inhibitors to noninhibitors was obtained for this system as compared with the γ G1-3-4 system. The additional proteins of the various subgroups described above were

also tested in the γ G2-4 system with parallel results (Table II). Only γ G2 proteins and the four γ G4 proteins discussed above were inhibitory in this system. Another antiserum made against one of these γ G4 proteins (He) gave exactly corresponding results after absorption. Fc fragments of these γ G4 and γ G2 proteins gave positive results while all Fab fragments were completely negative.

Distribution in Normal Sera.—All normal sera were positive for the γ G1-3-4 antigen, as expected, since all γ G1 and γ G3 myeloma proteins were reactive.

TABLE III
The γ G2- γ G4 System with Inhibition by γ G2 Proteins and Certain γ G4 Proteins

Inhibitor	Inhibitor Protein Concentration mg/cc				
	0.12	0.03	0.008	0.002	0.0005
Tr (γ G1)	2	2	2	2	2
De (γ G1)	2	2	2	2	2
Sm (γ G2)	0	0	0	0	1
Ca (γ G2)	0	0	0	0	2
Vi (γ G3)	2	2	2	2	2
Jo (γ G3)	2	2	2	2	2
St (γ G4)	2	2	2	2	2
No (γ G4)	2	2	2	2	2
Da (γ G4)	2	2	2	2	2
La (γ G4)	2	2	2	2	2
He (γ G4)	0	0	0	0	1
Mo (γ G4)	0	0	0	0	1

Coat- γ G4 protein He (DBB).

Agglutinator—monkey anti- γ G2 Ne absorbed with pepsin FrII and γ G1 protein Sn, γ G3 protein Jo, and γ G4 protein St.

This was also true for the γ G2-4 antigen because it was found on all γ G2 myeloma proteins irrespective of genetic type. However, since γ G4 myeloma proteins varied with respect to both antigens, it appeared probable that the γ G4 in normal serum would also show variation and that this variation would be on a genetic basis. Attempts were made therefore to isolate the γ G4 fraction from different normal sera for antigenic analysis in these systems. In initial experiments considerable purification of the normal γ G4 protein was achieved by combinations of electrophoresis and DEAE chromatography. However, other subgroup contaminants remained, which in most instances made typing difficult. Considerably greater success was achieved by using papain-split γ G4 preparations and making use of the unique property, the fast migration of the γ G4 Fc fragments. Mixtures of myeloma proteins were split with papain and

separated by zone electrophoresis on pevikon. The fast Fc fragment was obtained with both antigenic types of γ G4 proteins and could be obtained free of γ G2 as well as other subgroup proteins, indicating that the method was feasible.

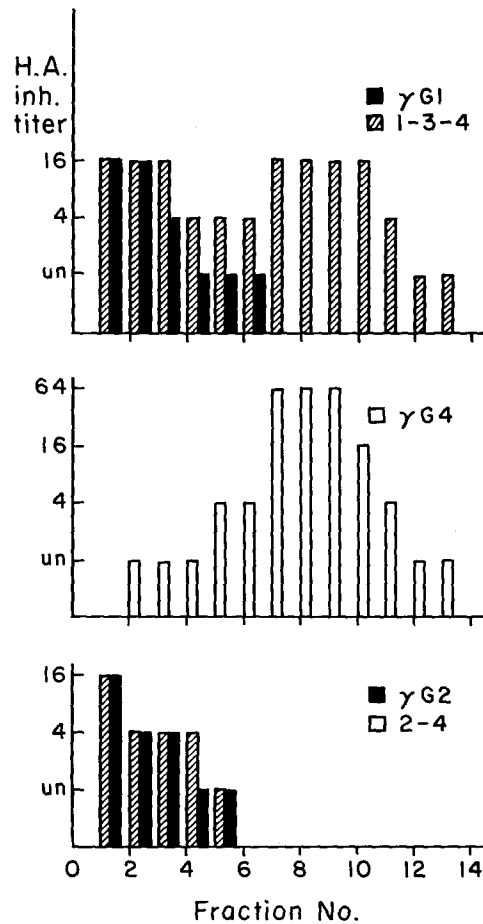


FIG. 1. Zone electrophoresis fractions after digestion of the γ -globulin from normal serum Te. The distribution of the γ G4 Fc is shown in the middle portion with little contamination from γ G1 and γ G2 Fc as determined by hemagglutination-inhibition titers. This serum is of the γ G1-3-4 type because the 1-3-4 antigen parallels the distribution of the γ G4 while the 2-4 antigen does not.

The γ G4 level in multiple normal sera was quantitated (9). As had been noted previously,² considerable variation was found; those sera with low levels (approximately 20% of the sera) were not utilized for further analysis. The

² Yount, W. J., and H. G. Kunkel. Unpublished observations.

remaining sera were separated by zone electrophoresis and the fractions containing peak quantities of γG_4 , usually in the fast γ - β area, were pooled and concentrated. Papain splitting of this material was carried out for 2 hr, a time which was found optimal for recovering γG_4 Fc without producing major

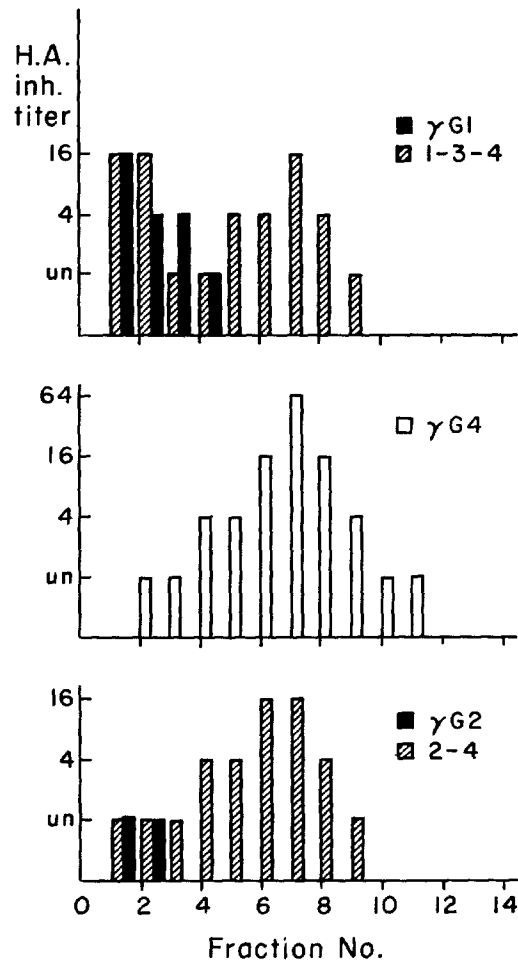


FIG. 2. Zone electrophoresis fractions from normal serum Lo. The γG_4 fractions from this serum contain both the 1-3-4 and the 2-4 antigens.

contaminants from other subgroups of interfering mobility. The material was then separated by zone electrophoresis and each fraction analyzed by hemagglutination inhibition for the various relevant antigens. Fig. 1 shows the results of one such study on the fractions of serum Te. Only the area showing

the γ G4 Fc is shown and peak levels are found in tubes 7–10. The results of analyses for γ G1 and γ G2 determinants show that these fall to zero before tube 7 and before the γ G4 peak, an essential criterion for a successful separation. The γ G1-3-4 antigen plotted at the top of Fig. 1 follows the γ G1 in the first fractions and then shows a clear peak corresponding to the γ G4 peak. The γ G2-4 antigen, shown at the bottom, follows the γ G2 closely and is completely absent under the γ G4 peak. Thus the γ G4 in this serum contains only the γ G1-3-4 antigen and completely lacks the γ G2-4 type. Analyses for γ G3 determinants showed these to be under the γ G1 fractions at very low levels. Other antigens of the γ G1, 2, or 3 classes, including the Gm markers, were also measured and were negative under the γ G4 peak.

The results for serum Lo are shown in Fig. 2. Again the γ G1-3-4 antigen peaked over the γ G4 area well beyond the contaminating γ G1. In addition, however, this serum showed a clear γ G2-4 peak over the γ G4 area. Thus the γ G4 in this serum represented a mixture of types with both antigenic types present.

30 sera were separated in similar fashion and the fractions analyzed for γ G1-3-4, γ G2-4, as well as control antigens. In a few instances either poor separation or too low levels of γ G4 prevented a conclusive typing. Table IV shows the results for 24 sera where unambiguous results were obtained. Most of the sera contained only the γ G1-3-4 antigen. However, in six instances the sera contained both the γ G1-3-4 and γ G2-4 antigens. In one serum, Br, only the γ G2-4 type was found. Since a variation on a genetic basis was suspected, the sera were selected so that primarily those homozygous at the γ G1 and γ G3 loci were tested. It is apparent from the table that the γ G2-4 antigen was only found in the sera that were Gm(f), Gm(b), Gm(n) and were absent in the Gm(a), Gm(g) types. Calculation of the significance of the difference gives a P value of < 0.01 for this small series.

The results for a few other sera are also shown in Table IV. Si, a Negro, and Ch, a Chinese both contained only the γ G1-3-4 antigen. Studies of Br were consistent with the interpretation that this individual was homozygous for the 2-4 gene (termed 4b) and that most of the individuals shown in Table IV were homozygous for the 1-3-4 gene (termed 4a). Six individuals were heterozygotes. Since the evidence indicated a linkage to the Gm system, the two genes have been called Gm^{4a} and Gm^{4b} .

DISCUSSION

The results of these studies demonstrate the presence of two types of γ G4 proteins, the common 4a(γ G1-3-4) and less common 4b(γ G2-4). They differ in antigens of the Fc fragment which are shared with the other subgroups. The 4a type contains an antigen shared with all γ G1 and γ G3 proteins and the 4b type shares an antigen with all γ G2 proteins. The possibility might be raised

that the minor group, the 4b proteins, are not really of the γ G4 class but perhaps represent some variant type of γ G2. The following points represent some of the evidence against such a concept. (a) Two different antisera typed these proteins in a positive fashion as γ G4. (b) After papain digestion they showed the unique fast Fc component characteristic of γ G4 proteins. (c) The proteins

TABLE IV
The Results of Analyses for the γ G1-3-4 and γ G2-4 Antigens in the γ G4 of Sera of Different Gm Phenotypes

Serum	Gm type	1-3-4	1-3-4 and 2-4	2-4
Jo	a-g-f+b+n+	+		
Sc	"	+		
Lo	"		+	
Ro	"		+	
Ko	"		+	
So	"		+	
Kr	"		+	
Br	"			+
Be	a-g-f+b+n-	+		
Jo	"	+		
Wi	a+g+f-b-n-	+		
Wa	"	+		
Fo	"	+		
Sa	"	+		
Ns	"	+		
Nf	"	+		
De	"	+		
Dj	"	+		
St	"	+		
Fa	a+g+f+b+n+	+		
Sp	"	+		
Ta	"		+	
Si	a+g-f-b+n-	+		
Ch	a+g-f+b+n+	+		

lacked all the known Gm genetic markers; the non a and non g antigens, found in all γ G2 and absent in γ G4 proteins, were also missing. (d) The proteins failed to fix complement after aggregation with BDB, a selective characteristic of γ G4 proteins. In view of these as well as other findings cited in the results, it appears clear that the 4a and 4b types share virtually all the selective characteristics of the γ G4 class. This evidence also argues strongly against the remote possibility that the 4b type might represent a fifth class of γ G globulin.

In order to determine if the two types represent genetic variants of the γ G4

class of proteins it was necessary to isolate the γ G4 fraction from normal serum. This was essential because each of the antigens was also found in certain other γ G classes without variation. Isolation was accomplished by separation of the γ G4 Fc fragments utilizing their unique rapid electrophoretic mobility. Accurate serum typing proved feasible and was carried out on a limited number of sera. Both the 4a and 4b antigens were found alone or in mixtures in different sera and the accumulated evidence obtained indicated that they represented genetic variants controlled by allelic genes linked to those of the Gm system. Fig. 3 illustrates in a diagrammatic fashion the γ G heavy chain genes for the constant area with the positions of the γ G4 markers. Three types of gene complexes encountered in the present study are illustrated. The finding that the 2-4 or 4b variant was only associated with Gm(b) and Gm(f) forms the primary basis for the associations illustrated. Without the use of isolated myeloma pro-

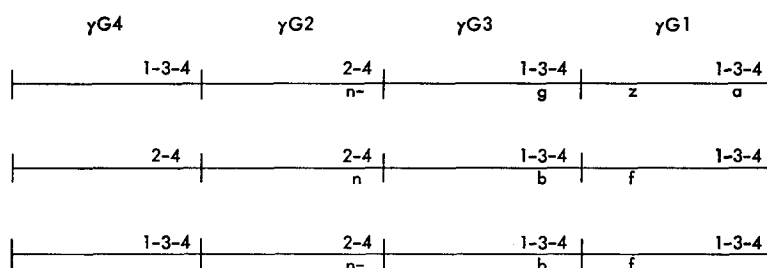


FIG. 3. Three common gene complexes for the four classes of γ G globulin showing the positioning of the 1-3-4 and 2-4 markers with respect to the known Gm genetic markers.

teins and the subsequent isolation of the γ G4 Fc fragment from normal sera, the γ G4 genetic variants would not have been apparent. It seems probable that similar variants may exist for other γ -globulins in various species that have not been recognized. Direct immunization of one animal with another animal's γ -globulin, as is usually done in searching for genetic variants, would not have uncovered the γ G4 variants.

The unusual feature of the γ G4 system that has not been encountered previously in genetic studies of the γ -globulins resides in their reciprocal relationship to the other classes of γ G globulin. The 4a marker was found in all γ G1 and γ G3 proteins but was absent in those of the γ G2 class, while the 4b marker was only present in the latter and absent in γ G1 and γ G3 proteins. If the two markers are considered individually, they might be thought of in the category of the non a and non g antigens described previously (4) which represent genetic antigens in one class but not in another. These markers were interpreted as antigens resulting from amino acid sequences shared between classes which were subject to independent mutations within a given class. Recent sequence studies (10)

have supported this interpretation. However, the two antigens obviously have to be considered together and some other explanation must be sought for their reciprocal relationship.

One interesting possibility is that the γ G4 class represents a relatively early evolutionary form of γ G globulin and that the allelic genes coding for the constant region of proteins of this class preceded the development of the genes for the other classes. The latter is presumed to have occurred through a process of gene duplication. Thus the gene for the γ G1 and γ G3 classes would have arisen from duplication of the 4a gene and the genes for the γ G2 class from duplication of the 4b gene. There are, however, certain findings that are difficult to fit in with such a hypothesis. For example, there is evidence for shared antigens between the γ G2 and γ G3 classes which are not found in the others; the non g represents one of these.

Most workers have considered the development of genetic variants as a process proceeding after gene duplication (10). There is no doubt that this is usually the case in various systems, including that for the γ -globulins. However, Herzenberg and associates (11) have demonstrated in the mouse system common genetic determinants which are shared by two classes of γ G globulin. These workers raised the possibility that gene duplication might have occurred after the mutations which led to the allelic genes, even though it was necessary to postulate repeated duplications to give all the combinations of alleles that were encountered. In addition, the finding of many antigenic and amino acid differences between proteins of a single γ -globulin class coded for by allelic genes has presented certain difficulties in interpretation. For example, the Fc portion of the Gm(b) and Gm(g) heavy chains of the γ G3 class differ by at least five separate antigens, some of which have been related to amino acid differences. It seems probable that, when complete sequence data becomes available, in certain instances greater differences will be found for genetic variants within a class than between the classes themselves. There is also evidence that some of the genetic antigens are not of recent origin and are present in a number of other primates. This is particularly true of the Gm(b) markers which are widely distributed among primates relatively distant from the human in the evolutionary scale (12, 13). Studies are currently underway to isolate and analyze different γ G classes in other primates with emphasis on the γ G4 type. Different human populations show very constant ratios for the concentrations of the four classes of γ G globulin which stand in marked contrast to the many very different genetic variants that are observed. The γ G4 system adds further complexity to these relationships. Our knowledge of just how the various genes coding for the constant area of the heavy chains became established remains fragmentary but it appears probable that these studies may offer some new clues.

SUMMARY

Two types of γ G4 proteins, termed 4a and 4b, were characterized through antigenic studies of myeloma proteins. Both were recognized by specific antigens on the Fc fragment which were shared with other γ G classes. The distinctive antigen of the common 4a type was shared with all γ G1 and γ G3 proteins but missing in those of the γ G2 class; that for the rarer 4b type was selectively found in proteins of the γ G2 class.

Analyses on γ G4 fractions isolated from normal sera showed that either the 4a or the 4b or a mixture of the two types was present in each serum. Evidence was obtained that these differences were on a genetic basis and that allelic genes linked to those of the Gm system were involved.

Such a reciprocal occurrence in other classes of γ G globulin of the antigenic markers distinguishing genetic variants has not been observed previously. A number of questions regarding the evolutionary development of the genes responsible are discussed. The possibility is raised that those for the γ G4 class arose relatively early and preceded the development of those for the other γ G classes.

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