GENETIC VARIANTS OF γG4 GLOBULIN

A UNIQUE RELATIONSHIP TO OTHER CLASSES OF γG GLOBULIN*

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The γG4 class of γ-globulin has aroused special interest because it differs most strikingly from the other γG 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 γG4 protein as compared with the major γG1 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 γG3 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 γG4 proteins have been described thus far.

The present studies were undertaken after a number of antigenic differences were noted among γG4 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 γG4 myeloma proteins also related to antigens shared with other γG 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 γG4 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 γG1-3-4 and γG2-4 Antigens.—Hemagglutination inhibition techniques were utilized as described previously (4). Two antisera were utilized primarily for the γG-1-3-4 antigen. The first was made in a rabbit against the γG1 protein Gr and the second was made in a cynomolgus monkey against the γG4 protein Ge. Both antisera were absorbed with pepsin Fr II and two γG2 myeloma proteins. As the protein coat, either anti-Rh antibodies or myeloma proteins coupled to red cells by BDB were utilized (4).

For the γG2-4 antigen two antisera were utilized. Both were raised in cynomolgus monkeys; one was against the γG2 protein Ne and the other against the γG2 protein He. Only red cells coated with myeloma proteins were utilized in this system; various γG2 myeloma proteins were attached to red cells with BDB and either of the γG4 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 γG1-γG3-γG4 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 γG 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: γG2-γG3, γG1-γG2-γG3, γG2-γG3-γG1 Gm(f), and γG2-γG3 Gm(b). The last named, for example, has been termed "non g" since it occurs in all γG2 proteins and only in the Gm(b) type of γG3 proteins. It is absent in all γG1 and γG4 proteins and in the Gm(g) variant of γG3.

Another shared antigen similar to those described above is the γG1-γG3-γG4 antigen which was at first thought not to be unusual except that all γG2 proteins were completely negative. However, more extensive study of γG4 proteins indicated that not all contained this antigen. Table I indicates the results of one system used for detection of the γG1-γG3-γG4 antigen. An antiserum against a γG4 protein absorbed with two γG2 proteins plus pepsin-treated FrII was used as the agglutinator against anti-D coated red cells. Proteins of the γG1 and γG3 subgroups inhibited to low concentrations while γG2 proteins failed to inhibit. Most γG4 proteins inhibited in a fashion similar to the γG1 and γG3 types. However, definite exceptions were found and proteins He and Mo, shown in Table I, completely failed to inhibit. A total of 27 γG1 proteins of both Gm(f) and Gm(az) types all reacted similarly. Eleven γG3 proteins of Gm(b) and Gm(g) types also inhibited similarly. All of 11 γG2 proteins (Gm(n) and Gm(n−) types) were completely noninhibitory. Some of these other proteins are listed in Table II. 23 γG4 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 γG3 protein was used as the agglutinator. The antigen was localized to the Fc fragment of the γG4 protein as well as to those of the other subgroups. One γG4 heavy chain disease protein contained the antigen. No reaction was obtained with γM, γA, or γD proteins.

Special analyses were carried out on the four γG4 proteins that failed to react. They were clearly γG4 proteins by a wide variety of criteria. Two different γG4 antisera typed them as γG4 proteins both by precipitation and hemag-

TABLE I

Comparison between Proteins of Different Subgroups with Respect to Inhibition of Agglutination in the γG1-3-4 System

Inhibitor	Inhibitor protein concentration, mg/cc				
	0.12	0.03	0.008	0.002	0.0005
Tr (γG1, κ)	0	0	0	tr	2
De (γG1, λ)	0	0	0	1	2
Sm (γG2, κ)	2	2	2	2	2
Ca (γG2, λ)	2	2	2	2	2
Vi (γG3, λ)	0	0	tr	tr	2
Jo (γG3, κ)	0	0	0	1	2
St (γG4, λ)	0	0	0	0	2
No (γG4, κ)	0	0	0	tr	2
Da (γG4, κ)	0	0	tr	1	2
La (γG4, κ)	0	0	0	1	2
He (γG4, κ)	2	2	2	2	2
Mo (γG4, κ)	2	2	2	2	2

Coat–anti D He.

Agglutinator–monkey anti-γG4 Ge absorbed with pepsin FrII and γG2 proteins Sm and Ne.

glutination. They lacked the γG1-2-3 antigen, as do other γG4 proteins. They lacked the γG2-3 antigen found in all γG2 proteins. Each of the isolated proteins were subjected to papain digestion and the unique fast Fc component characteristic of γG4 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 γG4 proteins, including the two under special study, failed to fix complement.[1]

The γG2-γG4 Antigen.—Since the four γG4 proteins that lacked the γG1-3-4

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

antigen appeared similar to γG2 proteins in this respect, they were studied for other similarities. A monkey antiserum to the γG2 protein Ne was found to react with these four γG4 proteins after absorption with a γG1, γG3, and γG4 protein containing the γG1-3-4 antigen. Table III shows some of the results

TABLE II

Myeloma Proteins of Different γG Classes and Genetic Types

Protein	Subgroup and genetic type	γG1–3–4 antigen*	G2–4 antigen*
Tr	γG1 (f)	++	0
De	" (az)	++	0
Gi	" (az)	++	0
Sn	" (f)	++	–
Ja	" (f)	++	0
Ti	" (az)	++	0
Ba	" (f)	++	0
La	" (az)	++	0
Vi	γG3 (g)	++	0
Jo	" (b)	++	0
Jn	" (b)	++	0
Br	" (b)	++	0
Sm	γG2 (n)	0	++
Ca	" (non g)	0	++
Ne	" (n)	0	++
Sp	" (non g)	0	++
Th	" (non g)	0	++
St	γG4	++	0
No	"	++	0
Da	"	++	0
La	"	++	0
Ro	"	++	0
Ke	"	++	0
Le	"	++	0
He	"	0	++
Ma	"	0	++
Mo	"	0	++

The presence of the γG1-3-4 and γG2-4 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 γG1-3-4 antigen. Exactly the reverse relationship of inhibitors to noninhibitors was obtained for this system as compared with the γG1-3-4 system. The additional proteins of the various subgroups described above were

also tested in the γG2-4 system with parallel results (Table II). Only γG2 proteins and the four γG4 proteins discussed above were inhibitory in this system. Another antiserum made against one of these γG4 proteins (He) gave exactly corresponding results after absorption. Fc fragments of these γG4 and γG2 proteins gave positive results while all Fab fragments were completely negative.

Distribution in Normal Sera.—All normal sera were positive for the γG1-3-4 antigen, as expected, since all γG1 and γG3 myeloma proteins were reactive.

TABLE III

The γG2-γG4 System with Inhibition by γG2 Proteins and Certain γG4 Proteins

Inhibitor	Inhibitor Protein Concentration mg/cc				
	0.12	0.03	0.008	0.002	0.0005
Tr (γG1)	2	2	2	2	2
De (γG1)	2	2	2	2	2
Sm (γG2)	0	0	0	0	1
Ca (γG2)	0	0	0	0	2
Vi (γG3)	2	2	2	2	2
Jo (γG3)	2	2	2	2	2
St (γG4)	2	2	2	2	2
No (γG4)	2	2	2	2	2
Da (γG4)	2	2	2	2	2
La (γG4)	2	2	2	2	2
He (γG4)	0	0	0	0	1
Mo (γG4)	0	0	0	0	1

Coat–γG4 protein He (DBB).

Agglutinator–monkey anti-γG2 Ne absorbed with pepsin FrII and γG1 protein Sn, γG3 protein Jo, and γG4 protein St.

This was also true for the γG2-4 antigen because it was found on all γG2 myeloma proteins irrespective of genetic type. However, since γG4 myeloma proteins varied with respect to both antigens, it appeared probable that the γG4 in normal serum would also show variation and that this variation would be on a genetic basis. Attempts were made therefore to isolate the γG4 fraction from different normal sera for antigenic analysis in these systems. In initial experiments considerable purification of the normal γG4 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 γG4 preparations and making use of the unique property, the fast migration of the γG4 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 γG4 proteins and could be obtained free of γG2 as well as other subgroup proteins, indicating that the method was feasible.

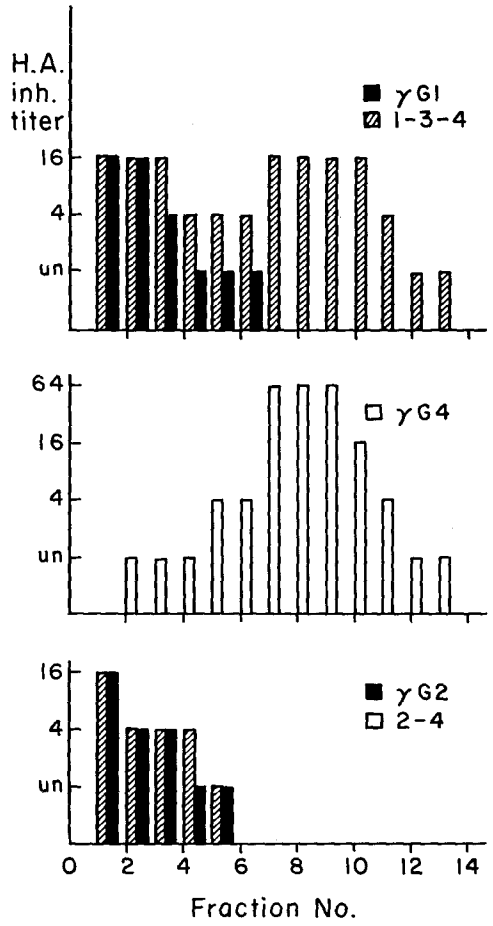

FIG. 1. Zone electrophoresis fractions after digestion of the γ-globulin from normal serum Te. The distribution of the γG4 Fc is shown in the middle portion with little contamination from γG1 and γG2 Fc as determined by hemagglutination-inhibition titers. This serum is of the γG1-3-4 type because the 1-3-4 antigen parallels the distribution of the γG4 while the 2-4 antigen does not.

The γG4 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 γG4, 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 γG4 Fc without producing major

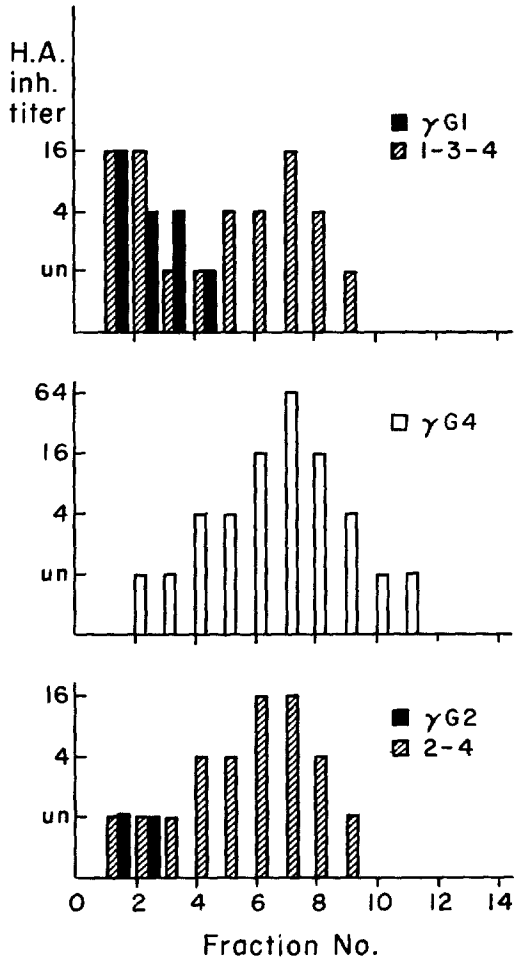

FIG. 2. Zone electrophoresis fractions from normal serum Lo. The γG4 fractions from this serum contain both the 1-3-4 and the 2-4 antigens.

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 γG4 Fc is shown and peak levels are found in tubes 7–10. The results of analyses for γG1 and γG2 determinants show that these fall to zero before tube 7 and before the γG4 peak, an essential criterion for a successful separation. The γG1-3-4 antigen plotted at the top of Fig. 1 follows the γG1 in the first fractions and then shows a clear peak corresponding to the γG4 peak. The γG2-4 antigen, shown at the bottom, follows the γG2 closely and is completely absent under the γG4 peak. Thus the γG4 in this serum contains only the γG1-3-4 antigen and completely lacks the γG2-4 type. Analyses for γG3 determinants showed these to be under the γG1 fractions at very low levels. Other antigens of the γG1, 2, or 3 classes, including the Gm markers, were also measured and were negative under the γG4 peak.

The results for serum Lo are shown in Fig. 2. Again the γG1-3-4 antigen peaked over the γG4 area well beyond the contaminating γG1. In addition, however, this serum showed a clear γG2-4 peak over the γG4 area. Thus the γG4 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 γG1-3-4, γG2-4, as well as control antigens. In a few instances either poor separation or too low levels of γG4 prevented a conclusive typing. Table IV shows the results for 24 sera where unambiguous results were obtained. Most of the sera contained only the γG1-3-4 antigen. However, in six instances the sera contained both the γG1-3-4 and γG2-4 antigens. In one serum, Br, only the γG2-4 type was found. Since a variation on a genetic basis was suspected, the sera were selected so that primarily those homozygous at the γG1 and γG3 loci were tested. It is apparent from the table that the γG2-4 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 γG1-3-4 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 γG4 proteins, the common 4a(γG1-3-4) and less common 4b(γG2-4). They differ in antigens of the Fc fragment which are shared with the other subgroups. The 4a type contains an antigen shared with all γG1 and γG3 proteins and the 4b type shares an antigen with all γG2 proteins. The possibility might be raised

that the minor group, the 4b proteins, are not really of the γG4 class but perhaps represent some variant type of γG2. The following points represent some of the evidence against such a concept. (*a*) Two different antisera typed these proteins in a positive fashion as γG4. (*b*) After papain digestion they showed the unique fast Fc component characteristic of γG4 proteins. (*c*) The proteins

TABLE IV

The Results of Analyses for the γG1-3-4 and γG2-4 Antigens in the γG4 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 γG2 and absent in γG4 proteins, were also missing. (*d*) The proteins failed to fix complement after aggregation with BDB, a selective characteristic of γG4 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 γG4 class. This evidence also argues strongly against the remote possibility that the 4b type might represent a fifth class of γG globulin.

In order to determine if the two types represent genetic variants of the γG4

class of proteins it was necessary to isolate the γG4 fraction from normal serum. This was essential because each of the antigens was also found in certain other γG classes without variation. Isolation was accomplished by separation of the γG4 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 γG heavy chain genes for the constant area with the positions of the γG4 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-

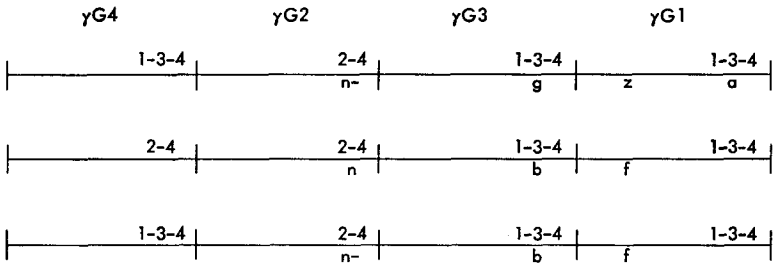

FIG. 3. Three common gene complexes for the four classes of γG globulin showing the positioning of the 1-3-4 and 2-4 markers with respect to the known Gm genetic markers.

teins and the subsequent isolation of the γG4 Fc fragment from normal sera, the γG4 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 γG4 variants.

The unusual feature of the γG4 system that has not been encountered previously in genetic studies of the γ-globulins resides in their reciprocal relationship to the other classes of γG globulin. The 4a marker was found in all γG1 and γG3 proteins but was absent in those of the γG2 class, while the 4b marker was only present in the latter and absent in γG1 and γG3 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 γG4 class represents a relatively early evolutionary form of γG 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 γG1 and γG3 classes would have arisen from duplication of the 4a gene and the genes for the γG2 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 γG2 and γG3 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 γG 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 γG3 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 γG classes in other primates with emphasis on the γG4 type. Different human populations show very constant ratios for the concentrations of the four classes of γG globulin which stand in marked contrast to the many very different genetic variants that are observed. The γG4 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 γG4 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 γG classes. The distinctive antigen of the common 4a type was shared with all γG1 and γG3 proteins but missing in those of the γG2 class; that for the rarer 4b type was selectively found in proteins of the γG2 class.

Analyses on γG4 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 γG 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 γG4 class arose relatively early and preceded the development of those for the other γG classes.

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