EVIDENCE FOR RECENT DUPLICATIONS AMONG CERTAIN GAMMA GLOBULIN HEAVY CHAIN GENES*

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Considerable antigenic and biochemical evidence has accumulated indicating that the four heavy chain subclasses of γG immunoglobulins are related to genes of at least four different cistrons (1–3). There are also striking structural similarities and a very close genetic linkage indicating that the subclass cistrons probably developed through relatively recent gene duplications. These four cistrons are under independent genetic control, since the rate of synthesis of the four corresponding subclass proteins is very different. Furthermore, various crossover events appear to have occurred among these cistrons. A number of gene complexes in separate population groups differ in the relationship of multiple Gm markers, some of which are known to involve more than one specific amino acid, as for example Gm(a) and "non-a," and probably Gm(g), "non-g," and Gm(n) (4, 5). Also, the unique γG1 molecule, Gm(f^+a^+), in Mongoloid populations has an Fd part like one type of Caucasian heavy chain, and an Fc part which is like another Caucasian type (6, 7). This is again most easily explained on the basis of a single regular intragenic crossover.

Strong evidence for structural gene deletions probably resulting from unequal homologous crossover has recently been presented (5, 8). Also hybridization among γG cistrons has been demonstrated in an individual homozygous for a rare γG3–γG1 gene coding for a γG heavy chain consisting of an Fd part similar to γG3 and an Fc part similar to γG1 (8, 9). This hybrid molecule was also most easily explained as a result of a deletion due to an unequal homologous crossover. Crossovers within families have proven difficult to demonstrate, but there is some evidence for fresh recombination within one family (3). In the latter study two additional families were described which appeared to show new recombinations. Another family probably similar to these has been reported by others (10). The present study provides new data on the two additional families and the accumulated evidence, including studies with the two new markers non-a and non-g, points to a rare duplication gene

complex in these families. A preliminary report of these findings has recently been presented (11).

Materials and Methods

Sera.—The family sera used were kindly provided by Dr. T. E. Cleghorn, Mr. J. Harris, and Mr. P. Issitt. Sera from normal blood donors with known Gm phenotype served as controls.

DEAE Chromatography and Gel Filtration.—Isolation of γG globulin from 10 ml of serum was performed with DEAE-cellulose batch chromatography using 100 g of Whatman DE-52 cellulose in 0.01 M phosphate buffer, pH 7.6. For fractionation of Fab, Fc, and unsplit γG globulin, DEAE column chromatography with stepwise elution was used. Fab fragments and undigested γG globulin were eluted with 0.01 M and Fc fragments with 0.2 M phosphate buffer, pH 7.6.

Gel filtration was performed with Sephadex G-150 in phosphate-buffered saline, pH 7.3, employing a 3.2 × 87 cm column and elution velocity of 26 ml/hr with an upward flow. The methods are fully described elsewhere (12).

Enzyme Digestion.—Digestion of γG globulin with papain was performed for 18 hr according to the methods of Jefferis et al. (13) with an enzyme:substrate ratio of 1:100 (w/w) in 0.1 M phosphate buffer, pH 7.0, with 0.01 M EDTA/Na_2. When cysteine was added it was used in a concentration of 0.001 M.

Hemagglutination-Inhibition Tests.—Genetic typing was carried out by hemagglutination-inhibition methods with rabbit and monkey anti-Gm antisera (5). Typing for the subclasses and the non-a and non-g antigens was also done by hemagglutination inhibition (4, 8). Selected anti-Rh antibodies were employed for red cell coating for the γG1 and γG3 specific test systems, while myeloma proteins coupled to red cells by bisdiazotized benzidine were used for the γG2 and γG4 systems.

Immunoelectrophoresis and Density Gradient Ultracentrifugation.—This was carried out by standard methods as described elsewhere (12, 14).

RESULTS

Determination of Gm Markers and non-a in Whole Sera.—In the first family (H. W.) extended studies on members not previously available showed two children with the rare phenotype $Gm(z^+a^+x^+f^+b^-g^+n^+)$ (Table I) and one with $Gm(z^+a^+x^-f^+b^-g^+n^+)$. Since three children had a rare phenotype which indicated an unusual gene complex, a fresh recombination was unlikely. In contrast a gene complex $Gm^f\ Gm^{za}\ Gm^g\ Gm^{n+}$ could more readily explain the findings. Similarly, in the second family (I) the first cousin of the propositus had two children who also showed the same rare phenotype $Gm(z^+a^+f^+b^-g^+n^-)$. This again indicated regular inheritance of an unusual gene complex Gm^f $Gm^{za}\ Gm^g\ Gm^{n-}$ (Table I).

Considerable evidence has accumulated indicating that Gm^z and Gm^f are homoalleles (7, 15). Therefore their simultaneous presence on the same chromosome strongly suggests that some type of duplication of the γG1 gene had occurred. However, special methods were required to answer the question of how extensive the duplication might be and whether an entire new heavy

chain was produced. Since Gm^z and Gm^f relate to markers in the Fd part of the heavy chain, the question arose whether the Fc part of the molecule was also involved. Here the Gm(a) and non-a markers represent the products of homo-alleles in the γG1 gene (4, 16, 17) and studies were directed towards determining if in these families these alleles were also present on the same chromosome. Gm(a) is a regular γG1 genetic marker. However, non-a is distributed not only in Gm(a⁻) γG1 proteins, but also in all γG2 and γG3 proteins, ir-

TABLE I

The Phenotypes and Proposed Gene Complexes in Families H. W. and I.

	Phenotype Gm z a x f g b n	Proposed genotype
Family H.W.		
Father	+ + − + + + +	Gm^{za} Gm^f Gm^g Gm^n /Gm^f Gm^b Gm^{n-}
Mother	+ + + − + − −	Gm^{zax} Gm^g Gm^{n-}/Gm^{za} Gm^g Gm^{n-}
Children		
Propositus	+ + + + + − +	Gm^{za} Gm^f Gm^g Gm^n /Gm^{zax} Gm^g Gm^{n-}
J. W.	+ + − + + − +	Gm^{za} Gm^f Gm^g Gm^n /Gm^{za} Gm^g Gm^{n-}
E. H.	+ + + + + + −	Gm^f Gm^b Gm^{n-}/Gm^{zax} Gm^g Gm^{n-}
M. W.	+ + + + + − +	Gm^{za} Gm^f Gm^g Gm^n /Gm^{za} Gm^g Gm^{n-}
Family I.		
Father	+ + + + + + −	Gm^{zax} Gm^g Gm^{n-}/Gm^f Gm^b Gm^{n-}
Mother	+ + − + + + +	Gm^{za} Gm^f Gm^g Gm^{n-}/Gm^f Gm^b Gm^n
Child (propositus)	+ + + + + − −	Gm^{za} Gm^f Gm^g Gm^{n-}/Gm^{zax} Gm^g Gm^{n-}
Mother's sister	+ + − + + + −	Gm^{za} Gm^f Gm^g Gm^{n-}/Gm^f Gm^b Gm^{n-}
Child	+ + − + + + +	Gm^{za} Gm^f Gm^g Gm^{n-}/Gm^f Gm^b Gm^n
Child's husband	+ + − + + + −	Gm^{za} Gm^g Gm^{n-}/Gm^f Gm^b Gm^{n-}
2 grandchildren	+ + − + + − −	Gm^{za} Gm^f Gm^g Gm^{n-}/Gm^{za} Gm^g Gm^{n-}
1 grandchild	− − − + − + +	Gm^f Gm^b Gm^{n-}/Gm^f Gm^b Gm^n

In the sera showing an unusual phenotype the inhibition for Gm(z) and Gm(f) was of the same strength.

respective of their Gm type. This marker is present in all normal sera, but shows high quantitative levels when present in the major γG1 subclass. In preliminary hemagglutination-inhibition studies, individuals with the rare gene complexes within the families H. W. and I. had high non-a levels, indicating that this marker probably was present and associated with Gm (f) as well as Gm(za) and controlled by the same chromosome. Further evidence for this would be obtained by determining non-a on isolated γG1 fractions from Gm(z⁺a⁺f⁺b⁻g⁺) individuals, M. W. from family H. W. and P. I. from family I. These persons had one normal Gm^{za} γG1 gene lacking non-a, together with the rare gene complex. Thus all the non-a within γG1 would originate from the rare gene combination, Gm^f Gm^{za}.

Determination of non-a in the γG1 Fc Fraction.—Differences in the suscepti-

bility of the four subclasses with respect to papain splitting were utilized to obtain γG1 fragments that could be tested for the non-a marker without interference from γG2 and γG3 material. The γG globulin from each of the two individual sera, isolated by DEAE chromatography, contained all the four γG subclass proteins. This material was then digested by papain without cysteine for 18 hr at an enzyme:substrate ratio of 1:100. Under these conditions γG1 and γG3 proteins were entirely split, but γG2 proteins were not. After subsequent chromatography with Sephadex G-150 and phosphate-buffered saline, pH 7.3, as elution buffer, the material was separated into two peaks (Fig. 1). The material of the first peak, designated Fraction (Fr) 1 in Table II, corresponded to the elution position of unsplit γG globulins, and
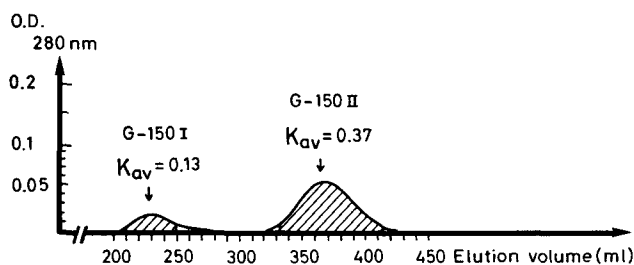

Fig. 1. Sephadex G-150 gel filtration of isolated γG globulin from serum P.I. after incubation with papain without cysteine. Unsplit γG2 proteins were found in Peak I and split γG1 and γG3 proteins were found in Peak II. For details see text. // indicate the fractions pooled.

immunoelectrophoresis tests gave no evidence for splitting. The material of this peak was negative when tested for the γG1 and γG3 genetic markers Gm(a), (b), (f), and (g) (Table II), and subclass-specific antigens related to γG1 and γG3. In contrast, γG2 Fc antigens were present along with non-a and non-g known to be present in all γG2 proteins; in the Gm(n)-positive individual H. W., Gm(n) was also present in this peak I material. Small amounts of γG4 proteins were also detected by hemagglutination inhibition with γG4 subclass-specific antiserum.

Both immunoelectrophoresis analysis and tests for genetic markers indicated that the material eluted between the two peaks showed some admixture of unsplit and split γ-globulin, and this material was not used for further studies. In contrast, peak II material contained a mixture of Fab and Fc fragments from the γG1 and γG3 subclasses. This was indicated by the presence of the Gm(a), (z), (f), (g), and non-a markers, while non-g and Gm(n) were absent. This lack of non-g showed that no contamination with γG2 proteins was detectable, and that the γG3 product of the rare gene complex was non-g negative. In some concentrated solutions the peak II material showed weak

inhibition in the γG4 system. However, since the γG4 subgroup does not contain any non-a activity this contamination could not influence the result.

The isolated peak II material was then incubated with papain (1:100) and cysteine (0.001 M) for 18 hr, designated Fraction II in Table II. This procedure completely cleaved the γG3 Fc fragments to small peptides which no longer

TABLE II

Results of Measurements for the Different Genetic Antigens in Fraction I and II Derived from Peaks I and II of Fig. 1. Fraction II Contains only γG1 Markers Including "non-a" Where This Is Present in γG1.

Subclass Gm	γG1			γG3		γG2	γG1-2-3	
	a	z	f	b	g	n	non-a	non-g
M.W. Gm(a+z+f+b−g+n+)								
FrI*	—‡	—	—	—	—	0.03	0.06	0.015
FrII	0.03	0.03	0.015	—	—	—	0.03	—
P.I. Gm(a+z+f+b−g+n−)								
FrI	—	—	—	—	—	—	0.015	0.008
FrII	0.015	0.015	0.008	—	—	—	0.03	—
Controls S.R. Gm(a−z−f+b+g−n+)								
FrI	—	—	—	—	—	0.015	0.03	0.015
FrII	—	—	0.015	—	—	—	0.03	—
E.F. Gm(a+z+f−b−g+n−)								
FrI	—	—	—	—	—	—	0.015	0.008
FrII	0.03	0.015	—	—	—	—	—	—
Pooled γG Gm(a+z+f+b+g+n+)	0.03	0.015	0.008	0.06	0.06	0.03	0.03	0.015

* Fr I material contains primarily unsplit γG2 with the antigens γG2 non-a, non-g, and Gm(n).

‡ Dash (—) indicates no inhibition in concentrations of 0.25 mg/ml for the fractions of serum M.W. and no inhibition at 0.5 mg/ml for all other fractions. For the inhibiting fractions the lowest concentrations giving inhibition are expressed in milligrams per milliliter.

carried genetic markers, as indicated by the hemagglutination-inhibition studies showing disappearance of Gm(g) activity as well as γG3 Fc activity. Also in control sera which were Gm(b)-positive, the Gm(b) markers of the γG3 subclass disappeared after such a procedure. Thus the material of peak II treated with papain and cysteine appeared to contain Fc fragments only of γG1 origin (Table II). This material showed a strong inhibiting activity both of Gm(a) and non-a in the sera M. W. and P. I. from the two families tested. In each case, Gm(a) and non-a activity was found to be of the same strength in this Fc γG1 material. In addition, the material also showed a strong inhibition for Gm(f) and Gm(z), showing that γG1 Fab fragments were still

present. In six normal control sera which were similarly treated, the presence of γG1 non-a was always associated with Gm(f), while Gm(f)-negative individuals always lacked γG1 non-a. The results obtained with two of these control sera are shown in Table II. For the critical family sera, isolation of the γG1 Fc and analysis for non-a activity was performed twice with the same result. In addition, the procedures were repeated for serum P.I. by using the DEAE-cellulose column technique instead of Sephadex G-150 for the separation of the γG1 and γG3 Fc material from unsplit γG2 and γG4 proteins after papain digestion. This method had certain advantages because the Fab fragments were also separated from the Fc (12). The γG1 and γG3 Fc material obtained was then subjected to papain digestion with cysteine, by the same method as previously mentioned, to break down the γG3 Fc. Again, the γG1 Fc material of serum P.I. showed strong Gm(a) and non-a activities, further demonstrating non-a in the γG1 Fc product of the rare gene complex.

Similar tests were also performed with sera from the two families, H.W. and I., of individuals carrying the unusual gene complex together with a regular Gm^f Gm^b gene complex. After splitting and isolation of the γG1 Fc fragment, as described above, the Gm(a) and non-a inhibiting activities were of the same strength in this material.

Additional Experiments.—Initially, the possibility had arisen that the Gm(a), (z), and (f) markers might be present on the same heavy chain through some type of duplication process producing a larger chain, as is known in the haptoglobin system. Evidence against this possibility, in addition to the non-a findings described above, came from gel filtration studies where the elution position on G-200 Sephadex of the γG1 proteins corresponded to that of normal 7S γG globulin. Further evidence in the same direction came from extensive density gradient ultracentrifuge experiments. The markers of the unusual Gm(a), (z), (f) complex sedimented exactly as did control γG1 markers which were supplied either from the normal chromosome of these heterozygous individuals or from other subclass markers in the same sera. The curves for the distribution of the determinants corresponded closely through the 7S region of the different gradients that were studied. It was clear that at least no large difference in the size of these proteins was present.

Attempts were also made to use immune adsorbent columns to demonstrate that the Gm(az) markers were on separate molecules from the Gm(f) marker in the unusual sera. The γ-globulin from antisera specific for Gm(f) were used as adsorbents by the technique of Avrameas and Ternynck (18). In control heterozygous sera, Gm(f) proteins were selectively removed, leaving Gm(az) proteins in the effluent. This was also found to occur in the unusual sera where all the Gm(f) came from the Gm^{za} Gm^f complex. The evidence obtained indicated that the Gm(az) and Gm(f) were on separate molecules. Similar results were obtained with anti–non-a antisera which were usually stronger than the

Gm(f) type. Table III shows the results of one experiment. Absorption with the anti–non-a antiserum removed most of the Gm(f) without affecting the Gm(a) in serum P.I. Control studies with serum from a normal heterozygous individual gave the same result.

DISCUSSION

The series of experiments presented showed that the γG1 Fc fragments originating from the rare gene complex were positive for the non-a marker as well as the Gm(a) marker. The detection of γG1 non-a depended on thorough isolation of γG1 Fc fragments through several steps which separated it from

TABLE III

The Effect on Gm(f) of Passage over an Immune Adsorbent Column Containing non-a Antibodies. The Gm(f) Titer Was Lowered in the Effluent of the Special Serum P.I. as in the Control Serum Pe. Gm(a) Was not Affected.

			$\frac{1}{2}$*	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Test for Gm(f)	Pe	(serum)	0	0	0	0	2	2
		(effluent)	0	0	2	2	2	2
	P.I.	(serum)	0	0	0	0	2	2
		(effluent)	0	1	2	2	2	2
Test for Gm(a)	Pe	(serum)	0	0	0	2	2	2
		(effluent)	0	0	0	2	2	2
	P.I.	(serum)	0	0	0	1	2	2
		(effluent)	0	0	0	2	2	2

* Inhibition titers of sera and effluents starting with the operating dilution (1/100).

$Pe = Gm^{az}/Gm^{non-a\ f}$; $P.I. = Gm^{az}/Gm^{az;\ non-a\ f}$

antiserum = anti-γG2 Fc abs.

γG2 and γG3 fractions which also contain the non-a antigen. The final material tested did not contain γG2 or γG3 subclass or Gm markers. Besides this direct typing of the γG1 Fc fraction, such a conclusion was also indicated by the absorption experiments where insoluble anti–non-a was able to remove the Gm(f) antigen. In addition to Gm(a) and non-a, the rare γG1 gene complex also produced the Fd markers, Gm(z) and Gm(f). In all other known instances from studies on sera of different populations and on isolated myeloma proteins and their fragments, Gm(z) and Gm(f) behave as if they are controlled by homoalleles. Considerable evidence is available indicating that these two antigens are related to a single amino acid interchange (lys-arg) in position 214 from the N terminus of the heavy chain. Similarly, Gm(a) and non-a are related to two amino acid differences (Asp-Glu-Leu and Glu-Glu-Met) in the residues 356–358 respectively (4, 15–17).

The occurrence of the two pairs of ordinarily allelic markers Gm^z and Gm^f

as well as Gm^a and *non-a* on the same chromosome is shown for one of the families in Fig. 2. Such a unique distribution is difficult to explain on the basis of point mutation, but is readily explained on the basis of an unequal homologous crossover giving rise to a duplication of the γG1 cistron. While evidence for gene deletions (5) and hybridizations (8, 9) probably resulting from unequal homologous crossover has recently been obtained, this is the first demonstration of a gene duplication among the immunoglobulins that is probably of relatively recent origin.
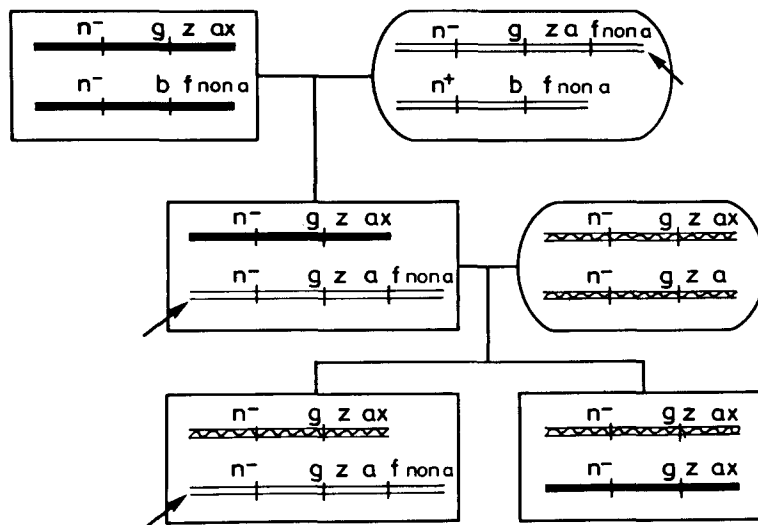
Gene Complexes. Family 1.

FIG. 2. Proposed gene complexes in family 1 (I) according to the phenotypes given in Table I and the non-a testing of isolated γG1 Fc. Non-a is only recorded for γG1 where it behaves as a genetic antigen antithetic to Gm(a). The proposed duplicated gene is apparent in three members of the family.

No evidence could be obtained for a partial duplication with one duplicated gene coding for one single larger polypeptide chain as is known for the haptoglobin system (19). No coprecipitation of Gm(z) or Gm(a) antigens was obtained when anti-Gm(f) and anti–non-a antisera were used. There was no indication of larger molecules by tracing the Gm(a), (z), and (f) markers in density gradient ultracentrifugation experiments. Thus the present evidence would indicate two independent γG1 genes on the same chromosome, Gm^{za} and $Gm^{f\,non-a}$ as shown in Fig. 3. These two γG1 genes probably combine with two variable genes to form two separate and complete heavy chains and two separate types of γG1 molecules. Since separate genes are involved new

mutations might occur in such a duplicated γG1 gene which might give rise to a different type of γG1 protein. Immunizations have been started in an attempt to detect antigens involved in such a possibility.

Much evidence has accumulated to support the theory that the heavy and light chain genes have developed through duplications of ancestral genes, and that the various heavy and light chain subclass genes are due to more recent duplications. The present detection of a recent gene duplication giving rise to two γG1 genes on the same chromosome is of importance since gene duplications appear to be the principal source of new genetic variability in higher vertebrates (20, 21). Thus the direct demonstration of duplicated γG1 cistrons in certain families is further evidence for the continued interplay of genetic forces in modifying the genome. Although the recombination rate
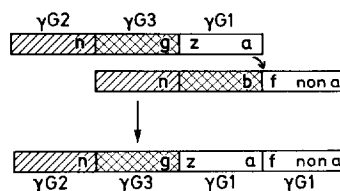

Fig. 3. Diagram of a postulated mechanism of gene duplication with mispairing of the γ-globulin genes followed by an intercistronic crossover at meiosis giving rise to two γG1 genes, one with Gm^{za} and another with $Gm^{f\ non-a}$.

among γG cistrons is extremely low, probably below 1/10,000, new recombinational events should be looked for particularly in these families because the misalignment during cell division or meiosis between a duplicated and normal gene complex may enhance further recombination (19). This study only includes markers and genes coding for the constant portion of the heavy chain where mutational events appear to be rare. However, similar genetic events at a greatly increased rate may also be operative in the variable region and may partly be involved in the generation of antibody diversity.

SUMMARY

A survey of a large number of human sera with the heavy chain genetic markers of the γ-globulin system has revealed an unusual gene complex which is inherited as a unit through two different families. The gene complex involves two pairs of γG1 genetic markers which ordinarily behave as homoalleles, Gm^z and Gm^f for the Fd part of γG1 molecules, and Gm^a and non-a for the Fc part. Isolation of the γG1 fraction from the unusual sera demonstrated the presence of the important non-a antigen in the γG1 fraction. Through the use of immunoadsorbents it was shown that these antigens were not part of a single molecule but that separate molecules were involved. The accumulated

evidence indicated that the appearance of such homoalleles on the same chromosome probably resulted from a recent gene duplication, giving rise to two γG1 cistrons on one chromosome.

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