Heavy-Chain Subclasses of Human γG-Globulin. Peptide and Immunochemical Relationships*

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ABSTRACT: Peptide maps and antigenic analyses were performed on human γG myeloma proteins representative of the four heavy-chain subclasses. Peptide analysis indicated that 17–22 of the 24 peptides found in the Fc fragment were shared between the subclasses. The γG_4 type showed the largest number of subclass-specific peptides.

On the basis of these results the subclasses could be ordered according to the degree of relationship to one another, as follows: γG_1, γG_2, γG_3, and γG_4. Data obtained from antigenic studies with rabbit and primate antisera confirmed this relationship.

Human γG-globulins have been subdivided into four heavy-chain subclasses, γG_1(We,2b), γG_2(Ne,2a), γG_3(Vi,2c), and γG_4(Ge,2d) on the basis of antigenic differences detectable by rabbit and monkey antisera (Grey and Kunkel, 1964; Terry and Fahey, 1964; Ballieux *et al.*, 1964). It has also been possible to relate these subclasses to the Gm allotypic markers: Gm, a, z, y, and f are found on γG_1-type molecules; Gm b and g on γG_3-type molecules; and Gm n on γG_2-type molecules (Kunkel *et al.*, 1964a; Terry *et al.*, 1965; Kunkel *et al.*, 1966). These findings have led to the concept that the production of the four different γG subclasses is controlled by four separate, closely linked genetic loci (Kunkel *et al.*, 1964b).

The technique of peptide mapping has been used recently to analyze the differences that exist between different Gm types (Meltzer *et al.*, 1964) as well as differences between heavy-chain subclasses of γG-globulin (Frangione *et al.*, 1966). Although differences in the peptide maps of different subclasses have been described by previous workers, no detailed study on the extent of the differences that occur between the different subclasses has been made. The present study of the peptide and antigenic structure of the four subclasses was undertaken in order to gain information regarding the extent to which the four heavy-chain subclasses are related to one another. Myeloma proteins representative of the different subclasses have been used in this study, since it has not been possible as yet to isolate the subclasses in a pure form from normal serum. While whole proteins have been used for antigenic analysis, peptide analysis has been limited to the Fc fragment portion of the heavy chain. This has been done to simplify comparisons between different proteins without the complication of the heterogeneity of that portion of the Fd fragment which differs between each myeloma protein (Frangione and Franklin, 1965; Grey *et al.*, 1965). The results indicate that all the subclasses are quite similar in structure and that there is an ordering of the subclasses with regard to the degree of relationship which, fortunately, is as follows: γG_1, γG_2, γG_3, and γG_4.

Materials and Methods

Myeloma Proteins. Myeloma proteins were isolated from the sera of patients with multiple myeloma by means of starch block electrophoresis (Kunkel, 1954). Isolated myeloma proteins were classified into one of the four heavy-chain subclasses by gel diffusion analysis, utilizing rabbit and monkey antisera specific for the different subclasses. Proteins were also classified according to their Gm types.[1]

Isolation of Fc Fragments. The isolated myeloma proteins (30–100 mg) were dialyzed against 0.1 M phosphate buffer (pH 7.5) and concentrated by negative pressure dialysis to a protein concentration of 20 mg/ml. The protein solutions were then made 0.01 M with respect to cysteine and 0.002 M with respect to EDTA. It was found that 0.01 M cysteine was sufficient to obtain good yields of Fc and Fab fragments, except in the case of γG_2 proteins where 0.1 M 2-mercaptoethanol was used, since preliminary experiments indicated that more drastic conditions of reduction than those provided by 0.01 M cysteine were required. Digestion with 1% papain (w/w) (Worthington, two times crystallized) was allowed to proceed for 1

* From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California, and The Rockefeller University, New York, New York. *Received April 13, 1967.* This is Publication No. 201 of the Department of Experimental Pathology, Scripps Clinic and Research Foundation. This investigation was supported by Research Grant AI-7007 of the U. S. Public Health Service, and in part, by Atomic Energy Commission Contract AT(04-3)-410.

† Department of Experimental Pathology, Scripps Clinic and Research Foundation. This study was done during the tenure of an Established Investigatorship of the American Heart Association.

[1] Gm typing was kindly performed by Dr. Stephan Litwin, The Rockefeller University.

TABLE I.

γG Subclass	Myeloma Protein	Gm Type	γG Subclass	Myeloma Protein	Gm Type
γG_1(We)	Bro	fy	γG_3(Vi)	Fel	b
	Car	za		Fra	b
	Chu	fya		Pre	b
	Gra	fy			
	Gre	za		Vil	g
	Ing	fy		Zuc	b
	Ken	za		(H chain protein)	
	Lan	fy			
	Lev	za			
	Pas	fy			
γG_2(Ne)	Carm	n	γG_4(Ge)	Fer	
	Dom	n			
	New	n		Ger	
	Spa	n			
	Thi	n			
	Bli	n		Her	

hr at 37°. Digestion was stopped by the addition of a fivefold molar excess of iodoacetamide. After 30 min at 4° the solution was dialyzed against cold saline. Fc fragments were isolated by starch block electrophoresis. Only those fractions that were free of Fab fragments as judged by immunoelectrophoresis with a rabbit anti-γG-globulin antiserum were pooled, dialyzed against water, and lyophilized.

Preparation of Tryptic Peptides. The lyophilized Fc fragments were dissolved in 8 M urea and reduced with 0.1 M 2-mercaptoethanol for 1 hr at room temperature. Alkylation with a 10% molar excess of iodoacetamide was allowed to proceed in the cold for 30 min. The protein solution was then exhaustively dialyzed against 0.2 M ammonium bicarbonate (pH 8.2). Digestion with trypsin was performed with a TPCK[2]-treated preparation[3] and the protein solution was digested with 1% (w/w) of trypsin for 16 hr at 37°. At the end of this period the peptide solution was frozen and lyophilized.

Peptide mapping was performed essentially as described by Katz *et al.* (1959). The lyophilized tryptic peptides (2 mg) were spotted on a sheet of Whatman No. 3 paper. Descending chromatography was performed for 16 hr at room temperature, using as solvent 1-butanol–acetic acid–water (3.4:1:5). Electrophoresis was then performed using a pH 3.6 pyridine–acetic acid–water (1:10:289) buffer, or a pH 6.4 pyridine–acetic acid–water (25:1:225) buffer. When the pH 3.6 buffer was used the peptides were electrophoresed for 70 min at 44 v/cm. When the pH 6.4 buffer was used they were electrophoresed for 100 min at 30 v/cm. The peptides were detected by dipping the paper in a 0.2% ninhydrin solution containing collidine (2,4,6-trimethylpyridine). Whenever there was sufficient material peptide maps were performed in triplicate and also stained for the presence of tyrosine, histidine, arginine, tryptophan, and sulfur-containing amino acids (Smith, 1960).

Elution of Peptides and Amino Acid Analysis. Triplicate peptide maps were made and one was stained with ninhydrin to identify the area in which the peptide to be eluted was located. This area was then cut out of the two unstained maps with the aid of marker peptides which fluoresced under ultraviolet light, and each cut-out area was eluted with 2 ml of water. The peptide maps were then stained with ninhydrin to determine whether the proper area was cut out. The eluted peptides were then lyophilized, hydrolyzed with 1.5 ml of 6 N HCl for 24 hr at 110°, dried in a desiccator over sodium hydroxide, and analyzed for amino acid composition on a Beckman Model 120C amino acid analyzer. Molar concentrations of amino acids were calculated, assuming 1 mole of lysine or arginine to be present in the peptide under analysis.

Results

In all the studies on the peptide maps to be detailed below, care was taken to run at least two maps on each protein and to have at least two different proteins chromatographed at the same time. A γG_1 protein was always run with each of the preparations of the other three subclasses. If, after staining for particular amino acids, there was still doubt as to the identity of certain peptides from different proteins, the two peptide mixtures in question were spotted in equal

[2] Abbreviation used: TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone.

[3] TPCK-treated trypsin kindly provided by Dr. R. Doolittle, University of California, San Diego.

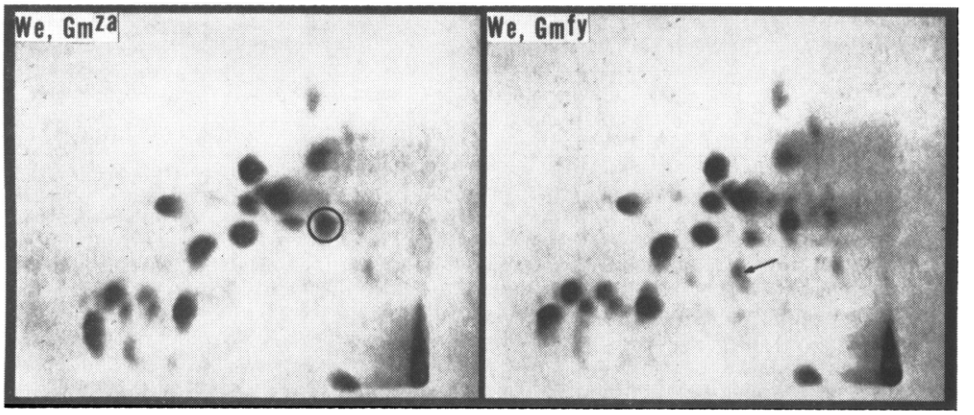

FIGURE 1: Peptide maps (pH 3.6) of γG_1 Fc fragments. (a) (left) Gm^{za} positive protein; (b) (right) Gm^{fy} positive protein. Spot marked by arrow in Gm^{fy} map was not found in the Gm^{za} map. Spot marked by circle on the Gm^{za} map is the probable location of the a peptide.

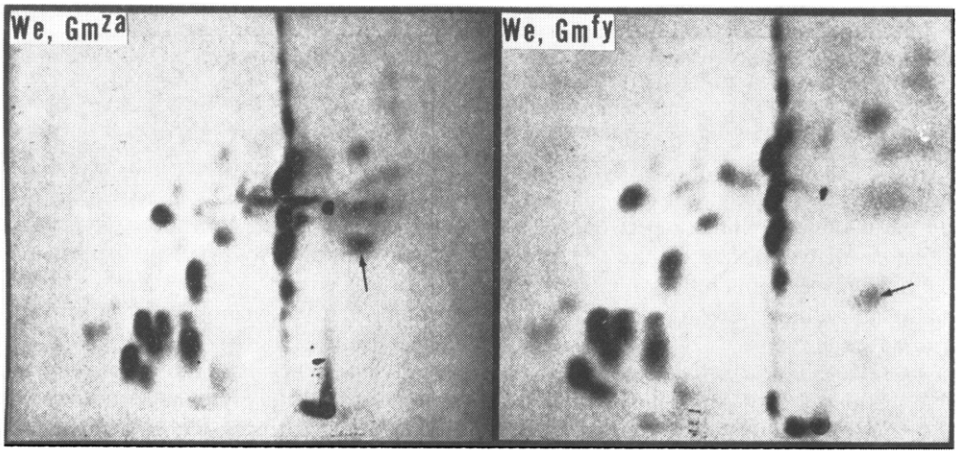

FIGURE 2: Peptide maps (pH 6.4) of γG_1 Fc fragments. (a) (left) Gm^{za} positive protein; (b) (right) Gm^{fy} positive protein. Spots marked by arrows refer to the position of the a and non-a spots, respectively.

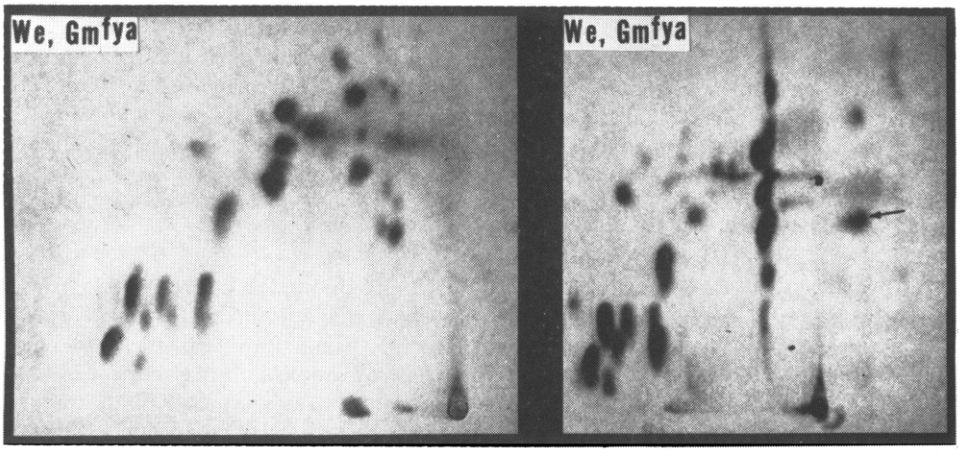

FIGURE 3: Peptide maps of mongoloid γG_1 Fc fragment with Gm^{fya} specificity. (a) (left) pH 3.6; (b) (right) pH 6.4. Note absence of non-a spot and presence of a spot (arrow).

amount on the same paper and the resulting fingerprint was examined for coincidence of the peptides under consideration.

Table I lists the myeloma proteins studied. A total of 24 proteins was examined, with proteins of all the subclasses and major Gm classifications being represented.

Peptide Maps of γG_1(We) Protein Fc Fragments. Peptide maps of all γG_1 Fc fragments were remarkably similar. When electrophoresis was performed at pH 3.6 there was a peptide present in the middle of the map (marked by the arrow in Figure 1b) of Fc fragments derived from Gm^{fy} positive myeloma proteins, which was absent in the maps derived from Gm^{za} proteins. At this pH no spot was seen in the Gm^{za} maps which was absent in the Gm^{fy} maps. The peptide marked with a circle in Figure 1a appeared to be somewhat more prominent in Gm^{za} maps and probably represents the area where the $Gm^{"a"}$ peptide was located at this pH, but apparently another peptide common to both Gm types was also present in this area and obscured the differences due to the Gm^{a} peptide. This point was clarified when the electrophoretic separation was done at pH 6.4 (Figure 2a,b). The overlapping peptides at pH 3.6 were separated at pH 6.4, making apparent the presence of a peptide in the Gm^{za} map, absent in the Gm^{fy} map ("a" spot), as well as the previously noted peptide that is present in the Gm^{fy} map and absent in the Gm^{za} map ("non-a" spot).

Mongoloid Gm^{fya} Myeloma Protein. A γG_1 myeloma protein from a Chinese patient with multiple myeloma which was typed as Gm^{fya} was also available for study. Figure 3 shows the peptide maps of this protein when electrophoresed at pH 3.6 and 6.4. The Gm^{a} spot was present in this mongoloid myeloma protein, whereas the non-a spot was absent. This correlated with the serologic typing of this protein as Gm^{a} positive. There were no peptide spots in this protein that could distinguish it from other caucasian Gm^{za} proteins. The possibility existed that the spot which appeared in the a spot position was different in its amino acid composition from the a spot found in Gm^{za} individuals; a spots were eluted from the Gm^{fya} map, as well as from a Gm^{za} map, and their amino acid composition was determined. Table II shows these results. There were no significant differences observed in the amino acid composition of the two peptides. The analysis of these peptides agrees with those previously published for the a spot (Frangione *et al.*, 1966; Thorpe and Deutsch, 1966), except for the higher values of valine and glutamic acid which were observed in both the caucasian and mongoloid a spot, presumably due to the elution of contaminating material.

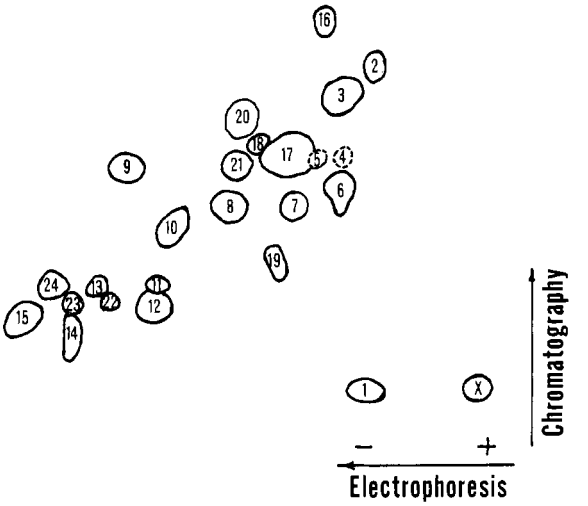

FIGURE 4: Tracing of peptide map (pH 3.6) of the Fc fragment from the γG_1 Gm^{fy} protein shown in Figure 1b. The peptides are numbered from 1 to 24. Those spots which coincided with free amino acids were omitted from the tracing.

Peptide Maps of γG_2(Ne), γG_3(Vi), and γG_4(Ge) Proteins. Figure 4 shows a tracing of the γG_1 Gm^{fy} map shown in Figure 1b. The 24 peptides were each given a number. Numbers 1–15 indicate peptides which were found in all four subclasses, whereas peptides 16-24 varied in their occurrence from one subclass to another.[4] Only those spots that were consistently observed in proteins of the γG_1 subclass were given a number. Certain peptides which were present in poorly resolved areas could be identified and distinguished from surrounding spots by the special sprays used to identify specific amino acids. For instance, peptide 5 gave a strongly positive reaction for tyrosine, even though it was often difficult to identify by ninhydrin;

TABLE II: Amino Acid Composition of Gm^{a} Peptide.[a]

Amino Acid	Caucasian Gm^{za} From Frangione *et al.* (1966)	Caucasian Gm^{za} Myeloma Car	Mongoloid Gm^{fya} Myeloma Chu[b]
Lys	1.0	1.0	1.0
Asp	1.2	1.6	1.3
Thr	1.1	0.9	1.0
Ser	0.4	0.6	0.7
Glu	1.4	1.9	1.7
Gly	0.4	0.5	0.5
Val		1.2	0.8
Leu	1.1	0.7	0.8

[a] Yields of amino acids are calculated as molar ratios, assuming 1 mole of lysine to be present. [b] Average of three separate elutions.

[4] Peptide maps of a mixture of free amino acids strongly suggested that free amino acids were present in many of the peptide maps. Spots present in the peptide maps which coincided with the spots obtained with free amino acids were omitted from all comparative analyses.

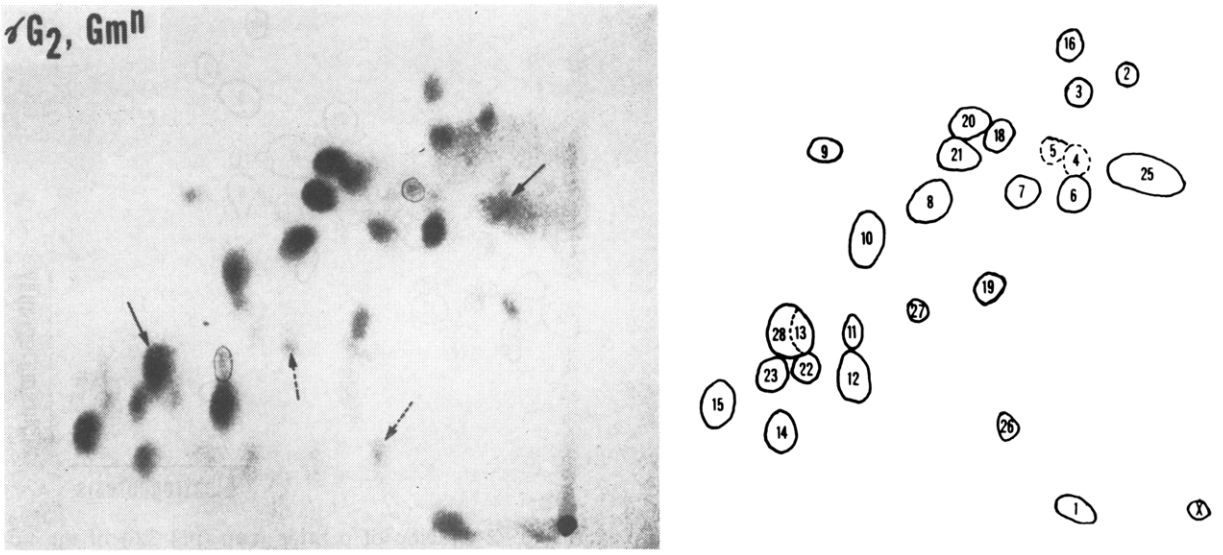

FIGURE 5: Peptide map (pH 3.6) and tracing of the Fc fragment from a γG_2 Gm^n protein. Numbers below 25 were in common to γG_1 and γG_2 maps. Arrows indicate spots present on γG_2 proteins which were not found on maps of γG_1 proteins. Dashed arrows refer to spots which coincide with peptides which occur in the Fc′ fragment after prolonged papain digestion.

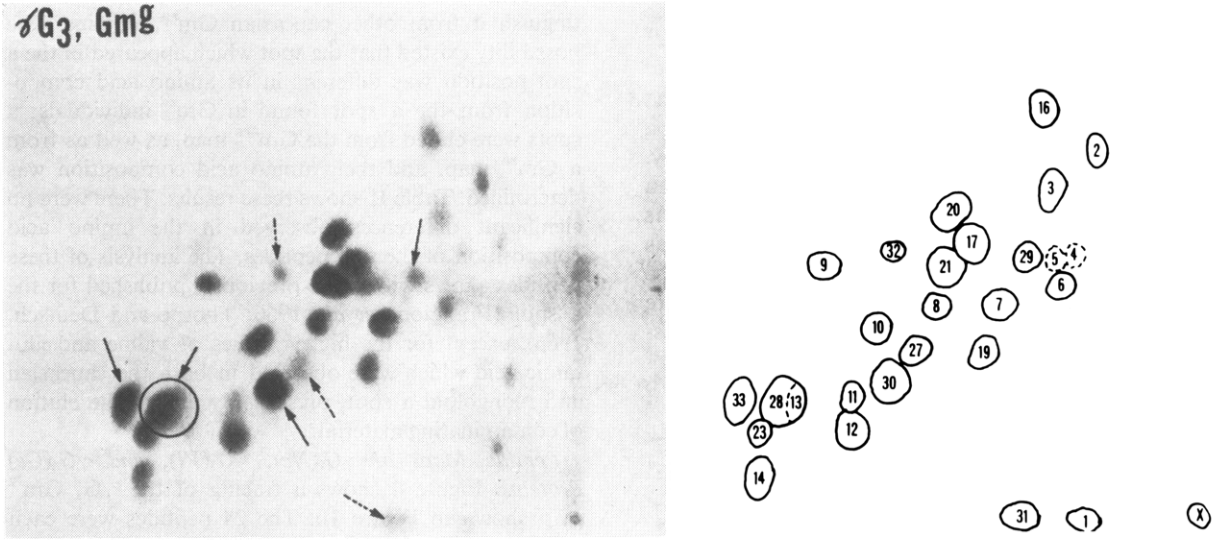

FIGURE 6: Peptide map (pH 3.6) and tracing of Fc fragment from a γG_3 Gm^g protein. Numbers below 25 were common to γG_1 and γG_3 maps. Arrows indicate spots present on γG_2 proteins which were absent on maps of γG_1 proteins. Circled spot was common to γG_3 and γG_2 proteins but was not seen on maps of γG_1 proteins. Dashed arrows refer to spots which coincide with peptides which occur in the Fc′ fragment after prolonged papain digestion.

peptide 4 contained arginine; peptide 18 contained tryptophan, which allowed its being identified as a separate peptide distinct from peptide 17; and spot 13 contained arginine, which allowed it to be distinguished from another, partially overlying, peptide observed in γG_2 and γG_3 proteins.

Six γG_2 Fc fragments were analyzed. All were Gm^n positive so that no data concerning the position of the Gm^n peptide could be obtained. The γG_2 Fc fragment was very similar to that of the γG_1 proteins (Figure 5). Of 24 peptides found in γG_1 Fc fragments, 22 were also present on all γG_2 maps studied. The numbered peptides in Figure 5 indicate the peptides common to γG_1 and γG_2 proteins. Peptides 17 and 24 were absent. In addition, γG_2 proteins had four peptides which were not found on γG_1 maps. Two of these spots (indicated by dashed arrows) were seen when peptide maps of the Fc′ fragment were made (Grey and

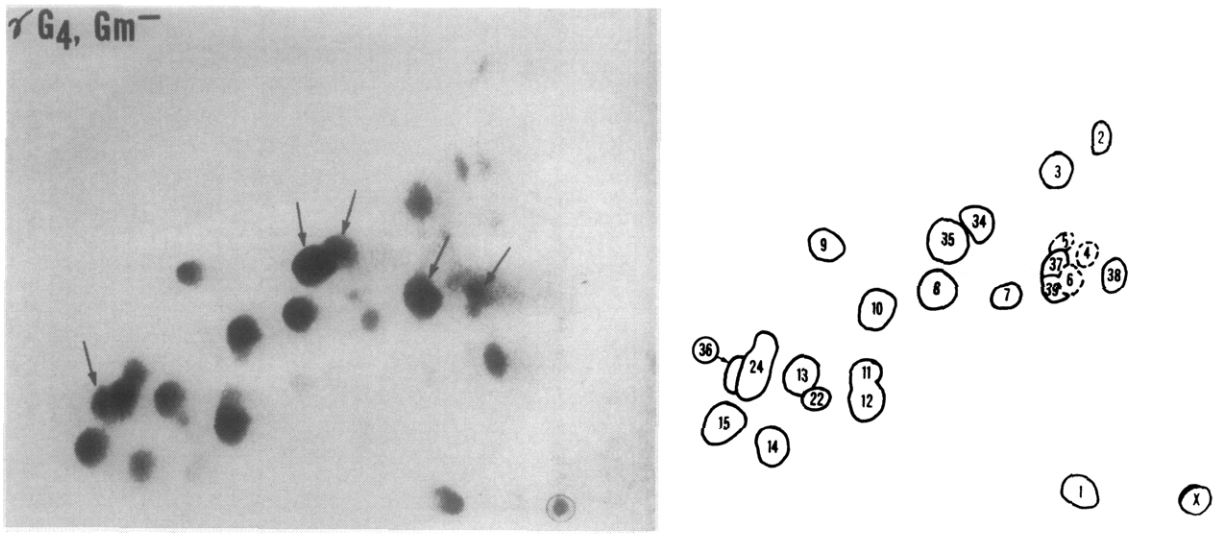

FIGURE 7: Peptide map (pH 3.6) and tracing of the Fc fragment from a γG_4 protein. Numbers below 25 were common to γG_1 and γG_4 maps. Arrows indicate spots present on γG_4 proteins which were absent on maps of γG_1 proteins.

Abel, 1967). The Fc′ fragment is a low molecular weight product obtained after prolonged papain digestion of γG-globulin which results from further enzymatic cleavage of the Fc fragment. Since those spots which are present in the Fc′ and absent in the Fc fragment arise from an artifact introduced by papain digestion, spots that by position and special stains appeared to be identical with peptides unique to the Fc′ fragment were omitted from comparative analyses.

Peptide maps of γG_3 proteins (Figure 6) were somewhat heterogeneous. However, there were some consistent differences between maps obtained with γG_3 proteins and γG_1 proteins. Three peptides present in γG_1 maps were absent on maps of γG_3 proteins (peptides 18, 22, and 24). Peptide 15 was also absent from this map but was present on all other γG_3 proteins studied. There were seven peptides present on the γG_3 maps which were not present on γG_1 maps. Of these seven peptides, three were likely to be the result of increased susceptibility of the γG_3 Fc fragments to papain digestion, for the same reason as stated above. When comparison was made between the maps obtained with γG_3 and γG_2 proteins, there was a difference of four peptides present in the γG_3 map which were absent in the γG_2 map, and three peptides present in the γG_2 map which were not found in the γG_3 maps. There was also a peptide present in both γG_2 and γG_3 maps (circle, Figure 6) which was absent in the γG_1 maps.

Peptide maps of the γG_4 proteins revealed the most differences when compared with the other subclasses (Figure 7). There were seven peptides present in γG_1 proteins which were absent in γG_4 proteins (peptides 16-21, 23), and six peptides present in γG_4 proteins which were absent in γG_1 proteins. The γG_4 maps differed from the γG_3 and γG_2 maps to a slightly greater degree than from the γG_1 maps.

The consistent differences found between all the subclasses are presented in Table III. From this table it can be seen that by the peptide mapping technique the subclasses can be ordered according to the degree of similarity to one another, so that the subclass closest to γG_1 is γG_2, whereas γG_2 is as related to γG_3 as it is to γG_1. γG_1, γG_2, and γG_3 differed from γG_4 proteins by 13, 15, and 18 peptides, respectively. Owing to the small number of γG_4 proteins available, the only conclusion that can be made at present is that γG_4 is approximately equally different from the other three subclasses. Whether γG_3 differs slightly more than γG_1 or γG_2 must await more complete studies.

Antigenic Relationship between γG Subclasses. Anti-

TABLE III: Tryptic Peptide Differences between γG Heavy-Chain Subclasses.[a]

Subclass	Total No. of Differences	Location of Spots Involved
γG_1 *vs.* γG_2	4	17, 24, 25, 28
γG_2 *vs.* γG_3	7	17, 18, 22, 25, 29, 30, 33
γG_1 *vs.* γG_3	(7)–8	(15), 18, 22, 24, 28, 29, 30, 33
γG_1 *vs.* γG_4	13	16–21, 23, 34–39
γG_2 *vs.* γG_4	15	16, 18–21, 23–25, 28, 34–39
γG_3 *vs.* γG_4	18	16, 17, 19–24, 28–30, 33–39

[a] Exclusive of those differences which were probably owing to over digestion with papain.

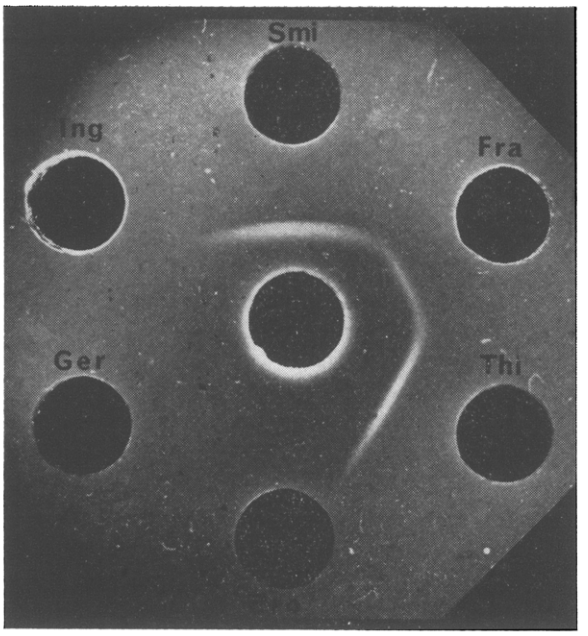

FIGURE 8: Ouchterlony analysis of subclasses. Center well: monkey anti-γG_2 γ-globulin absorbed with κ and λ chains and a γG_1 myeloma protein. Peripheral wells: myeloma proteins of the four subclasses Smi and Thi γG_2 proteins, Fra γG_3, Ing and Bro γG_1, Ger γG_4. γG_2 and γG_3 proteins reacted with lines of identity, whereas proteins of the other two subclasses failed to react.

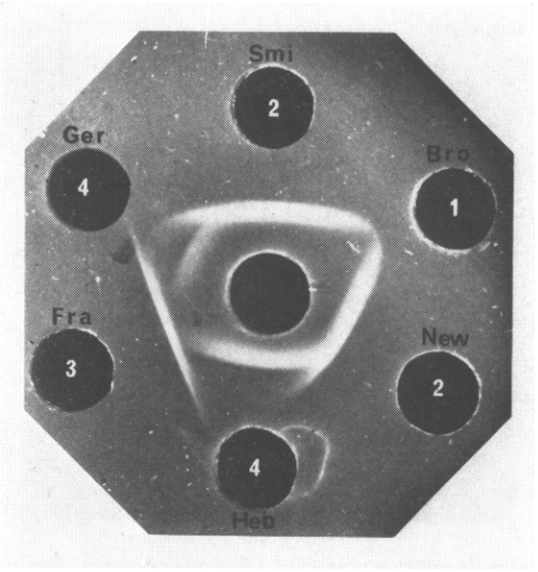

FIGURE 9: Ouchterlony analysis of subclasses. Center well: monkey anti-normal human γG-globulin absorbed with κ and λ chains and a γG_1 protein. Peripheral wells: myeloma proteins of the four subclasses Smi and New γG_2, Bro γG_1, Fra γG_3, Heb, and Ger γG_4. γG_2, γG_3, and γG_4 proteins reacted with lines of partial identity, whereas the γG_1 protein failed to react.

sera made in rabbits, as well as in primates, by prolonged immunization against the major γG_1 class of proteins always showed the γG_4 type to be most distantly related; strong spurs were obtained by Ouchterlony plate analysis of the γG_1 proteins over the γG_4 proteins with many antisera. Only a few of these antisera showed spurs of the γG_1 proteins over the γG_2 and γG_3 types. In these instances the γG_2 and γG_3 proteins in turn spurred over the γG_4 proteins. Antisera against the γG_2 and γG_3 proteins also showed a closer relationship between these proteins and the γG_1 class as compared to the γG_4 type. Specific antisera for the γG_3 class were more readily obtained in rabbits than for any of the other classes, primarily because of a unique determinant in the Fab portion of the molecule (Grey and Kunkel, 1964).

Primate antisera furnished evidence of antigenic determinants shared by the γG_2 and γG_3 classes which were absent in the γG_1 and γG_4 proteins. Antisera from several different primates made against the γG_2 proteins showed this specificity. Figure 8 illustrates the results with one of these antisera from the primate species *Papio leucophaeus*. Lines of identity between two γG_2 proteins and a γG_3 protein are shown, while no lines appear for γG_1 and γG_4 proteins. Similar findings were made with one baboon antiserum and two antisera from cynomologous monkeys, although with the latter antisera greater specificity for the γG_2 class was observed. Another antiserum prepared against normal γ-globulin in a primate of the species *P. leucophaeus* detected antigenic determinants common to the γG_2, γG_3, and γG_4 proteins which were absent in the γG_1 type (Figure 9). It also showed the γG_2–γG_3 common specificity mentioned above. When the antiserum was absorbed with γG_2 or γG_3 proteins, the reaction with the γG_4 proteins was removed.

Discussion

The above results on the comparison of the peptide maps of Fc fragments and the antigenic structure of whole myeloma proteins representative of the four human γG heavy-chain subclasses give information with regard to the nature of the relationships between the subclasses. Perhaps the most striking finding of the peptide maps of the myeloma protein Fc fragments was the similarity among all the subclasses. When comparisons were made between two different subclasses it was found that 75–90% of the tryptic peptides were held in common. This is undoubtedly a major factor involved in the difficulty in obtaining antisera specific for certain subclasses. Comparisons of the peptide maps of all four subclasses suggest the following relationships. γG_1 proteins are related to the other subclasses in the order $\gamma G_2 > \gamma G_3 > \gamma G_4$. γG_2 proteins

are related to the other subclasses: $\gamma G_1 > \gamma G_3 > \gamma G_4$. The order of relatedness to γG_3 proteins is: $\gamma G_2 \geq \gamma G_1 > \gamma G_4$, whereas to γG_4 proteins it is: $\gamma G_1 \approx \gamma G_2 \approx \gamma G_3$. If it is assumed, as it is for the hemoglobin peptide chains, that the different heavy-chain subclasses arose by the process of multiple gene duplication of a single ancestral gene, followed by separate evolutionary development of the duplicated genes (Ingram, 1963), the order in which the subclass genes duplicated and evolved would be directly related to the structural similarities between the peptide chains, and would be: γG_4, γG_3, γG_2, and γG_1 (or the reversal: γG_1, γG_2, γG_3, and γG_4). Genetic linkage studies should corroborate or disprove this hypothesis regarding the order in which the subclasses evolved if, following gene duplication, the genes remained linearly arranged on the chromosome.

Antigenic analyses, although subject to obvious limitations, in general substantiated the relationships found in the peptide map studies. The γG_4 subclass was most readily distinguished from the others and appeared deficient with many antisera made to the other proteins. A close relationship between γG_2 and γG_3 proteins was also apparent and these proteins shared determinants which were absent in the major γG_1 subclass. This relationship was most apparent with primate antisera. Other similarities and differences appeared through antigenic studies that were not obvious from inspection of the peptide maps.

It is of interest that the ordering of the subclasses on the basis of structural relationships (γG_1, γG_2, γG_3, and γG_4) is the same ordering that exists in terms of the relative quantity of the different subclasses present in normal human serum (Grey and Kunkel, 1964; Terry and Fahey, 1964). The significance of this correlation is not clear, but it may be indicative of an interrelationship between the subclasses with regard to control mechanisms involved in regulating their synthesis.

The present study corroborates the peptide mapping data of others (Frangione *et al.*, 1966; Thorpe and Deutsch, 1966) in localizing the peptides associated with Gm a activity, as well as confirming the close similarity between the peptide maps of the four subclasses. The a and non-a peptides were found in the same position as described by Thorpe and Deutsch (1966). However, there was a discrepancy between the results obtained by Frangione *et al.* (1966) and the present study when the electrophoresis was performed at pH 3.6. In the present study only the non-a peptide could be localized whereas the a peptide was overlapped by another spot, while in Frangione's study the a spot could be localized. This discrepancy is most likely due to the different methods employed for disulfide-bond cleavage. In the present study disulfide bonds were reduced and alkylated, whereas in the previous study the disulfide bonds were oxidized with performic acid. The a peptide lacks both cysteine and methionine, but the spot where the a peptide was suspected of being located gave a positive test for sulfur-containing amino acids. This has been interpreted as indicating that under conditions of reduction and alkylation the a spot is located in the same area as another peptide which contains sulfur, and that under conditions of oxidation this other peptide is localized elsewhere on the peptide map, thereby making the a peptide the only spot in that particular area of the map.

Considerable interest has been centered on the unusual combination of genetic markers found on certain myeloma proteins from mongoloid populations (Terry *et al.*, 1965; Martensson and Kunkel, 1965). Two hypotheses have been presented to explain the development of the Gm^{fya} gene that appear operative. These are (a) point mutation in the mongolian Gm^{fy} gene so that a new Gm^{fya} gene is formed; and (b) intragenic crossover between a Gm^{fy} and Gm^{za} gene to produce a Gm^{fya} gene. It was found that the Gm a spot from the mongolian Gm^{fya} protein had the same amino acid composition as the caucasian a peptide. Since the a peptide differs from the homologous peptide in the Gm^{fy} proteins by two amino acid residues, two separate mutations must be involved if mutation is considered to be the mechanism involved. This would appear to be a less likely possibility than that of intragenic crossover; although, since no unique "crossover peptide" was found which was not seen in caucasian Fc fragments, the crossover hypothesis requires further verification from sequence studies.

The methods used in this study, *viz.*, peptide mapping and antigenic analysis, both have certain disadvantages with regard to their use in the analysis of the structure of the heavy-chain subclasses. Because of the peptide variability in the Fd fragment portion of the heavy chain, peptide mapping was performed on the Fc fragment. It is known, however, that some subclass-specific antigens are present in the Fd fragment (Grey and Kunkel, 1964; Frangione and Franklin, 1965) so that peptide mapping of the Fc fragment incompletely analyzes that portion of the heavy chain responsible for subclass differences. Other disadvantages involve the variability of the products of enzymic digestion with papain. Papain digests proteins of the γG_3 subclass very rapidly and several peptides seen in maps of the γG_3 and γG_2 subclasses appear to be the same as some of those seen following prolonged papain digestion of γG_1 subclass proteins (Takatsuki and Osserman, 1964). Antigenic analysis, on the other hand, has the disadvantage that differences in antigenic structure may not reflect primary sequence differences but rather tertiary or quaternary structure differences. Also, the detection of antigenic differences is solely a function of the particular antisera available. Antigenic differences would not be detectable if the particular peptides involved are nonimmunogenic, due either to their chemical composition, position in the tertiary structure of the peptide chain, or because a state of tolerance exists due to the presence of similar or identical structures in the γ-globulin of the animal being immunized.

Acknowledgment

The technical assistance of Miss Mary Pat McNally is gratefully acknowledged.

References

Ballieux, R. E., Bernier, G. M., Tominaga, K., and Putnam, F. W. (1964), *Science 145*, 168.

Frangione, B., and Franklin, E. C. (1965), *J. Exptl. Med. 122*, 1.

Frangione, B., Franklin, E. C., Fudenberg, H. H., and Koshland, M. E. (1966), *J. Exptl. Med. 124*, 715.

Grey, H. M., and Abel, C. A. (1967), *Immunochemistry* (in press).

Grey, H. M., and Kunkel, H. G. (1964), *J. Exptl. Med. 120*, 253.

Grey, H. M., Mannik, M., and Kunkel, H. G. (1965), *J. Exptl. Med. 121*, 561.

Ingram, V. (1963), Hemoglobins in Genetics and Evolution, New York, N. Y., Columbia University.

Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.

Kunkel, H. G. (1954), *Methods Biochem. Anal. 1*, 141.

Kunkel, H. G., Allen, J. C., and Grey, H. M. (1964b), *Cold Spring Harbor Symp. Quant. Biol. 29*, 443.

Kunkel, H. G., Allen, J. C., Grey, H. M., Martensson, L., and Grubb, R. (1964a), *Nature 203*, 413.

Kunkel, H. G., Yount, W. J., and Litwin, S. D. (1966), *Science 154*, 1041.

Martensson, L., and Kunkel, H. G. (1965), *J. Exptl. Med. 122*, 799.

Meltzer, M., Franklin, E. C., Fudenberg, H., and Frangione, B. (1964), *Proc. Natl. Acad. Sci. 51*, 1007.

Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. 1, New York, N. Y., Interscience, p 95.

Takatsuki, K., and Osserman, E. G. (1964), *Science 145*, 499.

Terry, W. D., and Fahey, J. L. (1964), *Science 146*, 400.

Terry, W. D., Fahey, J. L., and Steinberg, A. G. (1965), *J. Exptl. Med. 122*, 1087.

Thorpe, N. O., and Deutsch, H. F. (1966), *Immunochemistry 3*, 329.