Note added in proof: Mr. Peter Moore at Harvard has independently determined that poly C and poly U compete for the same site (s) on ribosomes, and that the affinity of ribosomes for poly U is greater than their affinity for poly C. His measurements consist of sucrose gradient analysis of mixtures of ribosomes and labeled polynucleotides.

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*SINGLE PEPTIDE DIFFERENCES BETWEEN γ-GLOBULINS OF DIFFERENT GENETIC (Gm) TYPES**

By M. MELTZER, E. C. FRANKLIN,† H. FUDENBERG, AND B. FRANGIONE

NEW YORK UNIVERSITY SCHOOL OF MEDICINE AND UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE

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Genetically controlled polymorphism in human gamma globulins has been intensively studied since its initial recognition by Grubb eight years ago.[1] Similar genetic variations in gamma globulins ("allotypes") have also recently been demonstrated in other mammalian species.[2-4] Reagents to detect the various gamma globulin phenotypes of rabbits, mice, and guinea pigs can be produced by immunizing individuals of one genetic type with gamma globulin of another; allotypic differences are then readily detected by means of precipitating antibodies or by delayed hypersensitivity thus induced. In man, detection of genetic differences between individual gamma globulins has thus far been possible only by inhibition of more complex agglutination systems composed of rheumatoid factors (specific for one or another of the hereditary gamma globulin groups) and inert indicator red cells coated by incomplete antibodies from an individual of an appropriate gamma globulin type. (Rh positive cells coated by incomplete 7S anti-Rh are usually used.[1]) Each of the agglutinating systems thus established is inhibited by normal gamma globulin containing genetic determinants present in the antibody γ-globulin coating

the indicator red cells and complementary to the specificity of the rheumatoid factor. Many agglutinating sera of known specificity and 7S anti-Rh antibodies of known phenotype are available.[5] In general, the agglutinators are obtained by screening large numbers of sera from patients with rheumatoid arthritis or other diseases, and only little progress has been made in preparing reagents of one or another known specificity by immunizing infra-human species with human γ-globulin.[6] Sera or γ-globulins are phenotyped on the basis of their inhibitory effect upon such agglutination systems. A normal serum or the γ-globulin from it, which inhibits a given agglutination system, is classified as (+) for the factor detected by the system; if no inhibition occurs, the serum is (−) for the given factor detected by the test system. On the basis of such serologic studies, two nonlinked genetic loci, known as *Gm* and *Inv*, have been identified.[5] The alleles at these loci govern the production of the *Gm* and *Inv* serum factors.

Two main codominant alleles, (*a*) and (*b*), are present at each locus; at the *Gm* locus, a number of additional alleles, such as Gm^{ax}, Gm^{ar}, Gm^{abc}, have also been recognized which govern the production of *Gm* factors of lesser significance, such as *Gm*(*c*), (*e*), (*p*), (*r*), and (*x*).[5] If one considers only the two major alleles, three main genotypes exist in the *Gm* system: homozygous *Gm*(*a*)[*Gm*(*a*+*b*−)]; homozygous *Gm*(*b*)[*Gm*(*a*−*b*+)]; and the heterozygote *Gm*(*a*+*b*+). Similarly, three main genotypes exist for the *Inv* locus: homozygous *Inv* (*a*+); homozygous *Inv* (*b*+); and the heterozygote *Inv* (*a*+*b*+). It appears that individual molecules in the heterozygote possess one or the other, but never both characters determined by the same locus.[7] In addition, not all molecules can be accounted for by the factors currently known, suggesting that additional alleles exist.

In spite of the complex assay system and many unresolved questions, a great deal is now known about the structural basis for these genetic γ-globulin groups. Thus, *Gm* activity has been located only on 7S γ-globulin and, consequently, is confined to the F (B) fragment and the normal and pathologic A (H) chain, which carry the properties unique for this class of immune globulin.[8, 9] *Inv* activity, on the other hand, has been localized on the slow fragment (S or AC) produced by papain and on pathologic L chains (especially those of antigenic Type I[8, 9]). Consequently, since the slow (AC) fragment and L chains are common to all the normal immune globulins, *Inv* activity is found in each class of γ-globulin (7Sγ; 19Sγ; γ_{1A}; and γ_{μ}). Localization of *Inv* activity on artificially produced L (B) chains has not been possible in our hands to date,[10] presumably for technical reasons, but has been cited as a personal communication by Lawler in a manuscript by Cohen.[11] Similarly, the failure to detect *Inv* activity in Type II Bence-Jones proteins cannot be explained.

Since experience with other genetically determined variations in proteins in many species has indicated that in general these differences are based on well-defined differences in the primary structure of the proteins,[12-15] attempts were made to detect differences in the primary structure of γ-globulins of different genetic types employing a modification of the fingerprint technique of Ingram.[14] This has been successfully used in detecting amino acid difference between the various forms of hemoglobin[14] and haptoglobin[15] in man, and especially in detecting changes in some induced mutants of tobacco mosaic virus[13] and tryptophan synthetase of *E. coli*.[12] The findings indicate that γ-globulins with different genetic specificities differ in their

primary peptide composition and that the differences are limited to a single or a very small number of peptides.

Materials and Methods.—Sera: 20–30 ml fresh serum was obtained on one or more occasions from 21 healthy Caucasian donors, known to be homozygous ($a+$) or ($b+$) or heterozygous ($a+b+$) for the two major alleles at the *Gm* and *Inv* loci, and from one donor (Ripley) who is used as the standard anti-Rh coating serum for rheumatoid factor tests. This serum was kindly supplied by Dr. Marion Waller. Most of the sera were typed on several separate occasions with two separate sets of reagents for each factor with the exception of *Inv* (*b*) for which only one set of reagents is known.

Isolation of γ-globulin—fragments and chains: 7S γ-globulin was isolated by starch zone electrophoresis.[16] Purity was determined by immunoelectrophoresis using a variety of antisera to each of the immune globulins. All preparations contained more than 90% 7S γ-globulin and less than 10% of impurities. Papain fragments B(fast) and AC(slow) were prepared exactly as described previously.[17] A and B polypeptide chains were isolated from 7S γ-globulins by reduction in Tris pH 8.2 buffer with 0.025 or 0.75 *M* mercaptoethanol followed by separation on Sephadex G 100 in 1 normal acetic acid as described by Fleischman, Pain, and Porter.[18] All preparations were lyophilized prior to use.

Two-dimensional chromatography and electrophoresis (fingerprinting) was performed on performic acid oxidized fractions as described by Katz, Dreyer, and Anfinsen.[19] The lyophilized protein was suspended in formic acid (97+%) at a concentration of 40 mg/ml and then oxidized by performic acid in an ice bath at 0–2°C as described by Hirs.[20] The performic acid (9.5 cc of 97+% formic acid plus 0.5 cc 30% H_2O_2) was added to the protein solution in a ratio of 1.2 cc performic acid to 1.0 cc protein solution. The reaction was stopped after 2 1/2 hr by the addition of cold water to 50 times the original volume of the reaction mixture, then frozen and lyophilized twice. The lyophilized oxidized protein was suspended in an ammonium bicarbonate buffer, pH 8.4, 0.2 *M* to make a 2% solution. Twice recrystallized trypsin (Worthington Biochemical Corp., Freehold, New Jersey, batch 6126), 2% by weight, of the protein was added. Hydrolysis proceeded for 48 hr at 37°C at which time very little or no insoluble material remained. The hydrolysates were frozen and lyophilized. The lyophilized sample was suspended in the ammonium bicarbonate buffer (pH 8.4, 0.2 *M*) at a concentration of 100 mg/ml (1.0 mg in 10 μl). Either 2 or 3 mg samples were applied to Whatman 3MM filter paper (18 1/4 × 22 1/2) in a 6–8-mm diameter spot. For the first dimension, a descending chromatographic separation utilizing a butanol: acetic acid: water (4:1:5) solvent system proceeded for 21 hr. The paper was dried overnight at room temperature. The sheet was then sewn to another sheet of 3 MM filter paper and wet down with the buffer to be used for electrophoresis (pyridine: acetic acid: water, 1:10:289. pH 3.8). High-voltage electrophoresis in a direction perpendicular to that of the descending chromatography was done in a water-cooled tank under Varsol (Standard Oil Company, New Jersey) for 1 1/2 hours utilizing a potential gradient of 45 volts/cm (3,600 volts). The sheets were allowed to dry overnight at room temperature, then dipped in a 0.25% ninhydrin in acetone solution, and photographed after color had developed at room temperature after 48 hr.

Results.—Fingerprints were prepared on one or more occasions of the F fragments, A chains, or both, of 22 normal Caucasian adults of the genotypes listed in Table 1. In each F fragment preparation, about 25 well-defined spots were noted. In the A chains, three or four additional spots were generally, but not invariably, seen which appeared to be similar to spots present in maps of the slow (AC) fragment, but absent from maps of B chains. In addition to the spots in the F fragment, the A chains contained a number of faintly staining areas, some of which occurred in regions where specific differences between + and − proteins were apparent. Consequently, the detection of differences between *Gm*(+) and (−) fractions was somewhat more difficult in the chains than in the

TABLE 1

PREPARATIONS STUDIED

Genotype	No. of subjects	No. of F fragments	No. of A chains
Gm ($a+b-$)	5	2	3
Gm ($a-b+$)	9	9	4
Gm ($a+b+$)	8	6	5

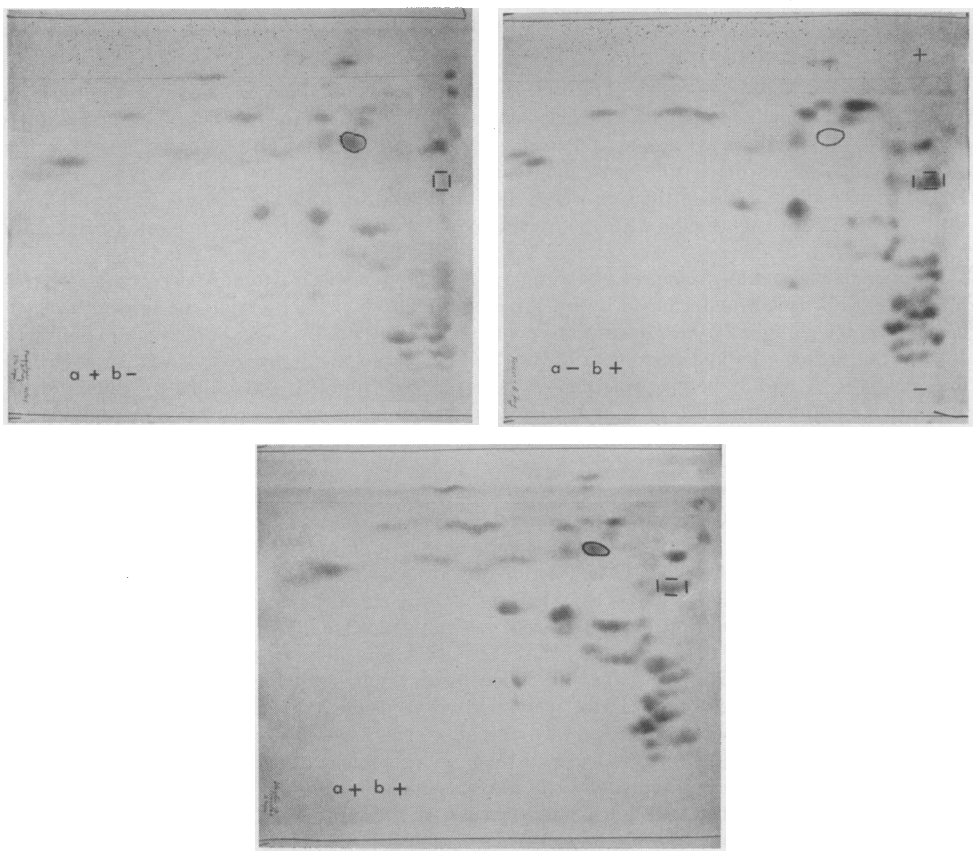

FIG. 1.—Fingerprint patterns of trypsin hydrolysates of F(B) fragments of γ-globulins of the three *Gm* types. The circle surrounds the *Gm* (*a*) spot; the square surrounds the *Gm* (*b*) spot. The three spots above the (*a*) region, and best seen in the (*a*−*b*+) pattern, varied from individual to individual but did not appear to be related to *Gm* (*a*, *b*, or *x*) activity.

fragments. However, their over-all structure appeared similar, qualitatively, and the results were identical whether A chains or F fragments were studied.

Figure 1 shows three representative fingerprints of F fragments prepared from 7S γ-globulins from *Gm* (*a*+*b*−), *Gm* (*a*+*b*+), and a *Gm* (*a*−*b*+) subjects. Although there were some variations in the relative intensity of some spots, especially those located just above the spot characteristic of *Gm* (*a*+) fragments, all the other spots appeared to be similar in all patterns with the exception of two areas. The area marked by a circle delineates the region where the *Gm* (*a*) differences were noted, and that marked by the square outlines the region where differences between *Gm* (*b*+) and (*b*−) proteins were visible. It is obvious from this figure that the peptide maps of F fragments of *Gm* (*a*+) γ-globulins had a dark spot which was absent in the map from the *Gm* (*a*−) fragment. Similar results were observed in eight (*a*+) F fragments, each of which had the spot, and nine (*a*−) F fragments which lacked it. In the *Gm* (*b*+) maps, two dark spots were visible of approximately equal intensity just directly below the origin. The upper one was present in all maps and was used as a constant reference marker, while the lower one was present in all of fifteen maps from *Gm* (*b*+) subjects, absent in one, and faint in a second

map of *Gm* (*b*−) individuals. Because of the relative scarcity of Caucasian *Gm* (*b*−) subjects, only five such proteins were available for study.

As shown in these figures, the differences were striking and easily recognizable, and involved only a single peptide spot for each allele. In each instance, with the exception of one map from a subject (Ripley) whose *Gm* (*b*) type could also not be accurately classified by serologic techniques employing a number of anti-*Gm* (*b*) reagents, the *Gm* type could be easily recognized from the peptide map by each of the authors without prior knowledge of the serologically identified *Gm* type.

Figure 2 shows three representative peptide maps prepared from A chains of the

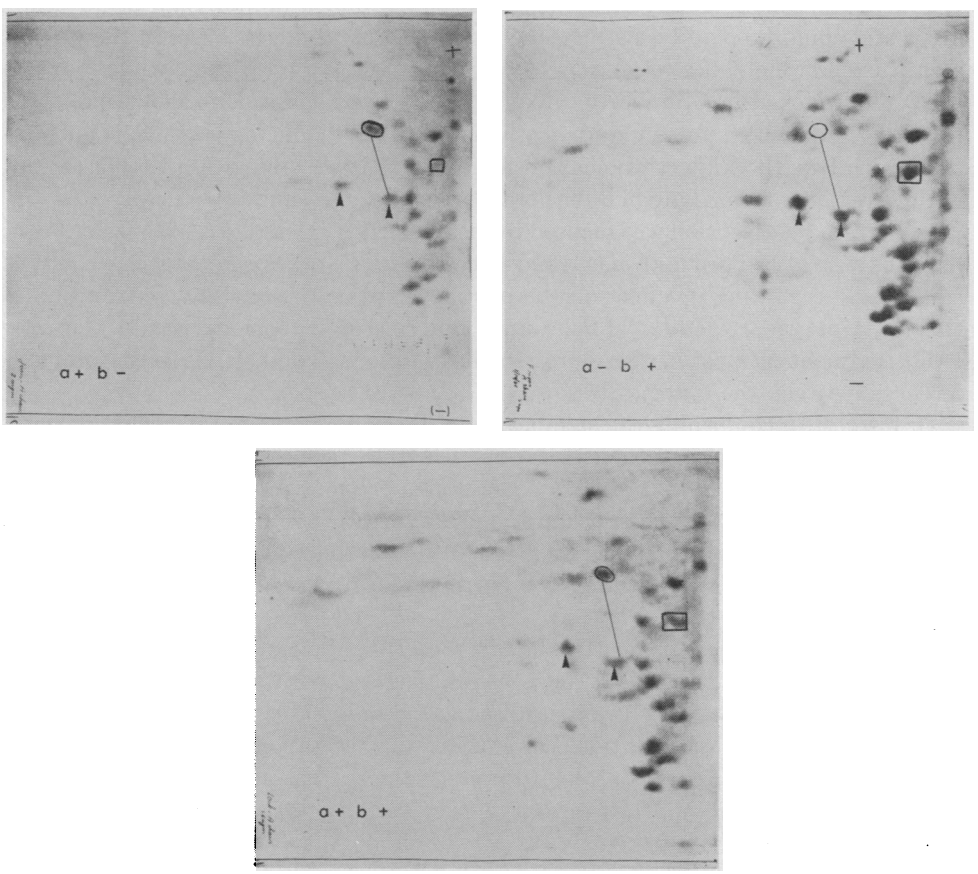

FIG. 2.—Fingerprint patterns of trypsin hydrolysates of A chains of γ-globulins of the three *Gm* types. The same variability in the three spots above the (*a*) region is again apparent.

same three genotypes. It is obvious that the same dark spot that had previously been identified in the *Gm* (*a*+) F fragments was present in the *Gm* (*a*+) A chain maps. However, an additional spot, slightly to the right of this, was usually present in all maps prepared from A chains. This spot could be readily distinguished when the two spots in question were viewed in relation to the two reference spots marked by the arrows. It can be seen that a vertical line could be drawn between the spot present in *Gm* (*a*−) maps and the reference spot beneath it. In the *Gm* (*a*+) maps, however, the dark spot characteristic of this *Gm* type could be connected to the same

reference spot by a line which formed an approximate 70–80° angle. In many *Gm* (*a*+) maps, both spots could be seen; however, the characteristic (*a*) spot was always darker and could be readily distinguished by an experienced observer. Again, as was the case in the F fragments, some variability in the intensity of the three spots just above the *Gm* (*a*) spot was noted which did not correlate with the *Gm* (*a*, *b*, or *x*) types.

In the case of the peptide spot unique for *Gm* (*b*+) subjects, the same dark spot previously identified as *Gm* (*b*+) in F fragments was visible also in *Gm* (*b*+) A chains. In the case of *Gm* (*b*−) A chains, however, faint staining was frequently visible in the same region. In every case this was much fainter than in *Gm* (*b*+) maps and could be readily distinguished with minimal practice. As in the F fragments, each of eight *Gm* (*a*+) maps had the characteristic (*a*) spot, while four *Gm* (*a*−) maps lacked it. Similarly, nine *Gm* (*b*+) maps had a dark spot while three *Gm* (*b*−) ones lacked it or showed only faint staining. Difficulty was again encountered with Rip, the subject who has been difficult to type serologically and has a spot in the *b* region intermediate in intensity between *Gm* (*b*+) and (*b*−) maps.

In this study, attention was focused primarily on *Gm* (*a*) and (*b*). However, most proteins were also typed for *Gm* (*x*), and since all *Gm* (*a*−*b*+) proteins are (*x*−), little can be said regarding its effect on the peptide maps or the relative contribution of the (*b*+) or (*x*−) factors. Fingerprint analyses of trypsin digests of the slow (AC) fragment of most of these proteins have failed to detect any structural differences between them, regardless of the *Gm* and *Inv* type.

Of interest in confirming the significance of these findings are the preliminary results of peptide analysis of the "H chains" naturally occurring in two subjects with "H chain" disease[21, 22] and six 7S γ-globulin myelomas. Both of the "H chain proteins" were *Gm* (*a*−*b*+) and had the characteristic *Gm* (*b*) spot and lacked the *Gm* (*a*) spot. All six myelomas were *Gm* (*a*+*b*−) and had the corresponding fingerprint patterns. These studies are currently being extended to other types of myeloma proteins.[23]

Discussion.—Studies correlating the fine structure of a gene with the corresponding protein controlled by it have progressed furthest with a number of microbial gene-protein systems.[12, 13] These studies have been greatly facilitated by the ease of mapping the sites of mutational alterations in these microorganisms. Further, the small size of the proteins produced permits the successful application of several techniques to the study of their primary structure. Similar correlations in man are more difficult to perform primarily because of an inability to induce mutations and to map their precise location on the gene. Analogous studies in man are of necessity restricted to polymorphic traits whose inheritance can be determined by family studies.

In the case of 7S γ-globulin, the available evidence suggests that synthesis of the part of the H chain and F fragment possessing *Gm* activity is under the control of alleles at the *Gm* locus. The ability to separate F fragments and A chains from 7S γ-globulin has simplified the studies of the primary structure of this protein in relation to its genetic control by allowing the removal of up to 50 per cent of the protein unrelated to this activity, and the performance of the appropriate studies on a somewhat less complex molecule with a molecular weight of about 50,000. However, similar results have recently also been obtained with the native proteins, thus pre-

cluding the possibility that the differences may be artifacts induced during the preparative procedure.[23]

The experiments reported clearly demonstrate the existence in tryptic hydrolysates of the appropriate fragments of both *Gm* (*a*+) and *Gm* (*b*+) subjects of a single peptide which cannot be seen in the *Gm* (*a*−) and *Gm* (*b*−) proteins. In the absence of precise amino acid analyses of this peptide or studies with other enzymes of different specificities, it is not possible to define the difference more precisely. From the known specificity of trypsin, it seems possible that the amino acid difference has given rise to an additional trypsin-sensitive bond, presumably one containing arginine or lysine. However, the possibility that the corresponding peptide in the *Gm* negative proteins is obscured by one of the other spots cannot be excluded. Studies are currently in progress to define more clearly the nature of this difference by employing other enzymes and by attempts to isolate the pertinent peptides in question and to analyze their amino acids. It seems possible, as is the case in bacterial systems, that the difference resides in a single amino acid substitution reflecting a change in a single base pair. The variability in the appearance of the *Gm* (*b*) spot suggests a greater degree of heterogeneity than in *Gm* (*a*) activity. This had been shown previously on the basis of serologic studies.[5,7]

This type of study and the techniques employed preclude accurate quantitative evaluation of the intensity of the spots and consequently do not permit any estimates concerning the number of molecules bearing a given characteristic in the homozygous as compared to the heterozygous subject; nor do they permit any statement concerning the effect of other minor *Gm* factors under the control of genes allelic to *Gm* (*a*) and (*b*).

Prior to the detection of the differences in the primary structures of γ-globulins of differing *Gm* types reported in these studies, differences in γ-globulin (*Gm*) types were detectable only by serologic techniques, based on differences in the antigenic properties of *Gm* (+) and *Gm* (−) γ-globulins. If the observed peptide differences are due to changes in single amino acids or a limited number in these peptides, it would suggest that these are the determinant groups responsible for the previously noted antigenic differences. Similar differences in antigenic properties reflecting only limited differences in single amino acids have previously been reported for some of the hemoglobins,[14] tryptophan synthetase of *E. coli*,[12] tobacco mosaic virus proteins,[13] and several others,[15] and will undoubtedly be found in other polymorphic systems. The availability of such peptides which can be isolated in pure form suggests their use as haptens to delineate the precise structure of the antigenic site.

In a broader sense, knowledge of genetically determined differences in the primary structure of γ-globulins is crucial in all studies directed to elucidate the nature of antibody specificity since such variations are constantly present in all animals. Any difference detected between purified antibodies, even if they are obtained from the same host, must always be viewed in the light of this underlying genetic heterogeneity. Since there is abundant evidence suggesting preferential involvement in a single antibody to a limited number of determinants of molecules of one genetic type,[24,25] comparison of two purified antibodies from a single animal with one another and with the remaining γ-globulin devoid of such antibody specificity is fraught with danger unless the subject is homozygous at each allotypic locus. Consequently, studies in inbred strains of animals where there is the least chance of genetic hetero-

geneity will be most likely to yield significant data on differences in the primary structure of purified antibodies.

Summary.—Fingerprint analyses of the F fragments and A chains of 22 subjects of different *Gm* types have demonstrated the presence of a peptide spot in $(a+)$ subjects which cannot be seen in $(a-)$ proteins, and another spot in $(b+)$ proteins which is lacking in $(b-)$ subjects. Other minor variations were occasionally seen, but did not correlate with the *Gm* (*a*, *b*, or *x*) type. The findings suggest that these differences reside in a single amino acid substitution reflecting a change in a single base pair.

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† Career Scientist, New York City Health Research Council # I 274.

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