STRUCTURAL STUDIES OF HUMAN IMMUNOGLOBULINS

Differences in the Fd Fragments of the Heavy Chains of G Myeloma Proteins*

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Plates 1 to 3

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Dissection of the G immunoglobulin molecules in man, using enzymic (1, 2) as well as chemical techniques (3, 4), has proceeded more rapidly than similar studies of the other two classes of Ig's and has provided a great deal of insight into the nature of the structural units and their genetic control. Using starch gel electrophoresis (3, 5) immunologic techniques (6, 7), and peptide analyses (8), it has been possible to demonstrate differences in light chains of different myeloma proteins, Bence Jones proteins, and antibodies. Similar techniques have also demonstrated differences in electrophoretic mobilities of the heavy chains of different myeloma proteins (5, 9). Except for a report showing that differences in the mobilities of myeloma proteins correlate with the properties of the Fd fragments (9), little is known about the exact nature of these variations or their possible significance. A precise delineation of these differences has been hampered by difficulties in obtaining the Fd fragment (A piece), free of light chains or the Fc (fast) fragment.

One approach to this question, which obviates the necessity of isolating the Fd fragment, is to determine its composition by comparing the peptide analyses (fingerprints) of the heavy chains and the Fc papain fragments from individual myeloma proteins or normal IgG fractions. Further information dealing with structural differences between different myeloma proteins, and differences between myeloma proteins and normal G immunoglobulins can be obtained by comparing the fragments and chains from different myeloma proteins to each other and to those obtained from normal G immunoglobulins. Since the Fd fragment (10) consists of that part of the heavy chain not present in the Fc fragment, it is possible to recognize the peptides belonging to the Fd fragment without actually having to isolate it by comparing the maps of these two readily available, well defined structural units.

The studies reported here have demonstrated a striking similarity in the peptide maps of the Fc fragments of a large number of normal and pathologic G immunoglobulins belonging to the We (b) antigenic subtype of γ-chains. Similarities were also noted among Fc fragments prepared from a smaller

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number of Vi (c) type of myeloma proteins. The major variations within each subtype were those related to differences in the Gm type previously reported (13); a few additional minor differences were occasionally noted, but these were generally absent from the heavy chains of the same protein. In contrast, the heavy chains from different G myeloma proteins, presumably by virtue of the Fd fragment, showed a great deal of variability and, generally, contained several spots not seen in the corresponding heavy chains from normal subjects. This finding, together with the observation that normal heavy chains contain a greater amount of faint background staining and fewer distinct well defined peptides than expected on the basis of arginine and lysine content, and also a smaller number than heavy chains prepared from individual myeloma proteins, suggests that much of the heterogeneity known to be associated with myeloma proteins, and probably also with antibodies of different specificities may reside in the Fd fragment.

Methods and Materials

Protein Fractions.—Normal G immunoglobulins and G myeloma proteins essentially free of IgA and IgM proteins were isolated by starch zone electrophoresis (14). Purity of all fractions was checked by immunoelectrophoresis. The "heavy chain" protein Zu was isolated from the urine and was kindly supplied by Dr. E. Osserman (15).

Papain fragments and polypeptide chains were prepared as described in reference 16. Fc fragments from 3 of 7 type c (Vi) proteins were difficult to obtain, even when digestion was stopped after 1 or 2 hours because they are readily destroyed by the enzyme (17, 18). Consequently, only 3 Fc fragments of the c (Vi) type were available for study.

Fingerprinting was done as described in (13, 16).

Immunologic studies were performed by double diffusion in agar or by immunoelectrophoresis. The antigenic subtypes of the heavy chains were initially determined by Dr. W. Terry and Dr. J. Fahey and, subsequently established in our own laboratory with antisera prepared against proteins typed by them and made specific by absorption with myeloma proteins of the other types.

More detailed studies of individual variations among myeloma proteins of the We (b) antigenic subtype were carried out with antisera to 6 of these proteins. These antisera were made specific for the heavy chains by absorption with a pool of Bence Jones proteins of type I and II, and failed to react with light chains prepared from the G myeloma protein used for immunization.

RESULTS

Fig. 1 illustrates a representative peptide map of the Fc fragment from a Gm (a+b+f+) subject, and the heavy chain from a normal individual who was Gm (a−b+f+). There are about 25 distinct dark spots, and a number of fainter ones in the Fc fragment. This is approximately one-half the number expected on the basis of the arginine and lysine content. In addition to these, there were 7 to 8 additional dark spots in the heavy chain. On the basis of the number of arginine and lysine residues estimated to be in the Fd fragment (5, 19), there should have been almost twice this number. In addition, several

fainter spots were noted. It appeared possible that this discrepancy might be due to the heterogeneity of the IgG fraction. If much of the variability in the structure of different antibodies and myeloma proteins were to reside in the Fd fragment, many peptides would be liberated in amounts too small to be detected and, consequently, one would expect to see fewer than the calculated number of dark peptide spots. In the absence of sufficient amounts of purified antibodies for analysis, the question was investigated by studying a large number of G myeloma proteins.

Figs. 2 to 5 compare the fingerprints of the Fc fragments and heavy chains prepared from 2 myelomas of the We (b) type and 2 myeloma proteins of the Vi (c) type. The fingerprints of the Fc fragments of all the myeloma proteins belonging to the same antigenic subtype were quite similar to each other. Within Fc fragments of the We type, variations appeared to be related primarily to the peptides associated with the Gm types (20). However, 3 of the Fc fragments of the We type (Ch, Tr, Ma) lacked the peptide shown by the circle in Fig. 2, and in 2 others (Bu, De), it was faint and displaced to the right. Since the missing peptide was present in each of the heavy chains from the same protein, it seems possible that this is simply due to technical differences.

Because of the greater susceptibility of the Fc fragments of the Vi (c) type to proteolysis by papain, comparative studies of these fragments and the heavy chains were possible in only 3 of 7 Vi (c) proteins. The overall appearance of these peptide maps (Figs. 4 and 5) was similar to that of the We (b) proteins (Figs. 2 and 3). However, in addition to the variations related to the Gm type, they had a somewhat different arrangement of some of the peptides at the bottom of the map, and generally lacked a peptide in the region marked by the circle. Since these differences were less striking in the heavy chains from the same proteins, their precise significance is difficult to evaluate at this time; it is possible that they may be related to the preparative techniques or the greater susceptibility of the fragments to papain digestion. This problem of the variations between different immunologic and genetic subtypes of γ-chains will be discussed more fully in a separate report (21).

In each instance where fragments were available, comparison of the heavy chain to the Fc fragment prepared from the same myeloma protein clearly demonstrated the presence of 6 to 13 extra spots in the heavy chain which were not seen in the corresponding Fc fragments. The results with 14 myeloma proteins of the We and Vi types are summarized in Table I. Detailed comparisons of these peptide maps to each other and to normal γ-chains reveal three major findings. Firstly, while some of these peptides appear in similar positions in maps from different γ-chains, the overall peptide distribution is different for each of the myeloma γ-chains examined, a finding which suggests a unique primary structure for each myeloma protein. Secondly, from 3 to 9 spots associated with the Fd fragments of the myeloma proteins were also

found in the normal Fd fragments. Since contamination to this extent seems unlikely, it seems probable that some of these peptides are present in a significant fraction of normal G immunoglobulin molecules. Studies of the light chains of 8 of the myeloma proteins clearly established that none of the extra spots could be due to the traces of light chain contaminants detected by immunologic techniques. Confirmatory evidence localizing more than half of the extra spots to the Fd fragment was obtained by studies of the Fab fragments from nine of the myeloma and a number of normal proteins. In each

TABLE I

Comparison of Peptide Maps of Heavy Chains of 14 Myeloma Proteins to the Homologous Fc Fragments and Normal Heavy Chains

Protein	No. of spots in Fd frag. (heavy chain-Fc frag.)	No. of spots in normal Fd frag.	No. of spots absent from normal Fd frag.
We (b) type			
De	11	7	4
Ch	13	6	7
Ma	8	4	4
Tr	13	6	7
Ca	7	3	4
Bu	9	4	5
Ba	6	3	3
Pr	11	7	4
Co	8	5	3
Na	14	7	7
Fr	9	7	2
Vi (c) type			
Gl	8	5	3
Ma	8	5	3
He	10	9	1

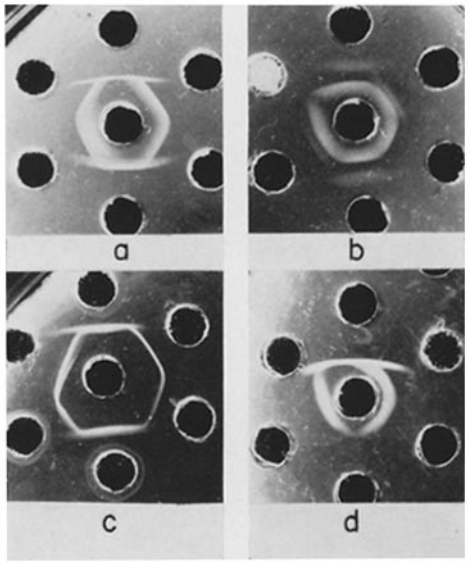
instance, from 5 to 10 of the Fd spots could also be recognized in the maps of the Fab fragments. Figs. 6 and 7 illustrate the peptide maps of the Fab fragments of 2 of the proteins (Pr and He) shown in Figs. 3 and 5. Comparison of these maps suggests that most of the peptides not contributed by the light chains are similar to the peptides attributed to the Fd fragments in the heavy chain peptide maps. Thirdly, detailed comparison demonstrated that virtually all of the myeloma heavy chains lacked a few (1 to 6) peptides present in normal heavy chains, and that they contained others (1 to 7) which were not detected normally. The possible significance of this finding will be discussed below.

Additional evidence pointing to the association of some of the unique properties of myeloma proteins with the respective Fd fragments came from studies

TABLE II

Localization on the Fd Fragment of the Antigenic Specificity of 3 Myeloma Proteins of the We (b) Type

Antiserum to	Antigenic Specificity		
	Native	Heavy chain	Fc fragment
Ma	+	+	−
Pr	+	+	−
Fr	+	+	−



TEXT-FIGS. 1 *a* to 1 *d*. Ouchterlony plates using antiserum to a myeloma of type b (We)-Pr in the center wells.

TEXT-FIG. 1 *a*. Homologous protein (Pr) (top and bottom wells) and 4 other We myeloma proteins in the side wells.

TEXT-FIG. 1 *b*. Homologous Fc fragments (top and bottom wells) and Fc fragments of 3 other proteins of the same type on the side. The faint outer lines are due to Fab fragment contaminants.

TEXT-FIG. 1 *c*. Homologous heavy chains (Pr) (top and bottom wells) and heavy chain of 4 other proteins of the same type on the side.

TEXT-FIG. 1 *d*. Fab fragment Pr top and 3 others of the same type laterally.

of their antigenic properties. Table II summarizes the results of immunologic analyses of myeloma proteins of the We (b) type with antisera to 6 of these proteins which had been absorbed with a pool of Bence Jones proteins till they no longer reacted with light chains. The precipitin lines of 3 of these antisera

with the homologous protein used for immunization formed significant spurs over each of 8 other myeloma proteins of the same antigenic type (Text-fig. 1 *a*). In contrast, 3 of the antisera failed to distinguish the proteins used for immunization. When the first 3 antisera were tested with the Fc fragments from 6 myeloma proteins, including the one used for immunization, they gave a reaction of complete identity (Text-fig. 1 *b*). In contrast, the heavy chains used for immunization resembled the native proteins in forming spurs over 4 other heavy chains (Text-fig. 1 *c*). Similar studies with the Fab fragments from the same proteins showed some degree of antigenic specificity with the fragment from the protein used for immunization in all 5 cases (Text-fig. 1 *d*). These findings clearly support the concept that some of the antigenic specificity of these proteins is also found in the Fd fragment of the heavy chain, but does not bear on the question of the antigenic specificity of light chains.

DISCUSSION

It is generally accepted that antibodies with different specificities differ from each other in primary structure (22), and that all myeloma proteins, like purified antibodies, are structurally unique (23). Earlier observations of myeloma proteins and antibodies emphasized the differences in light chains (3, 24). More recent studies concerned with the problem of antibody specificity have pointed to the role of the heavy chain in determining antibody specificity (25–29) and in reflecting differences between different myeloma proteins (5, 9, 12). Aside from the starch gel electrophoretic studies of myeloma heavy chains by Cohen (5), and the further localization of these differences to the Fd fragment by Fahey (9), little is known about the precise difference between different myeloma proteins and antibodies having different specificities.

The results of the present studies have bearing on several of these points. The peptide maps clearly demonstrate the similarity in the fingerprints not only of the Fc fragments of different myeloma proteins, but also their striking resemblance to normal Fc fragments. The observed differences in the maps of the heavy chains suggest that the unique peptides must reside in the Fd fragment, a finding consistent with the electrophoretic studies of Fahey (9). A more direct test with Fd fragments prepared by reduction and alkylation of Fab pieces, or pepsin digestion of heavy chains has not been possible because of our inability to obtain the Fd fragment free of either light chains or large non-dializable peptides. However, confirmatory evidence was obtained by the alternate approach of studying the antigenic properties of the Fab fraction with antisera absorbed with light chains. Here again antigenic specificity of the protein used for immunization was frequently noted, and shown to reside in the Fd fragment. This very limited localization of variability to only one-half of what is now considered the heavy chain, taken together with the occurrence in man of certain pathologic proteins resembling the Fc fragment (30), supports

the possibility that the Fd fragment may be a real structural unit of IgG. This concept is further strengthened by some of the results of genetic studies of rabbit γ-globulins (31, 32).

Of particular interest is the presence, in each of the myeloma heavy chains, of a number of peptides not detectable in normal heavy chains, and the absence of others characteristic of the normal Fd fragment. This finding lends further support to the idea that much of the heterogeneity of normal γ-globulin resides in differences in the Fd fragments. If this were the case, only those peptides present in a significant fraction of the molecules could be detected, while many others present in smaller amounts would not be recognized. This possibility is particularly attractive in view of the many studies suggesting that the Fd fragment is the region which contains the antigen-combining sites. A more definitive answer to this question will have to await the result of similar studies with purified antibodies.

SUMMARY

1. Comparison of peptide maps of the Fc fragments of normal G immunoglobulins and 11 G myeloma proteins of the We (b) type showed them to be very similar except for differences associated with the Gm type. Some additional differences were noted, however, in the Fc fragments of three Vi (c) myeloma proteins.

2. Peptide maps of heavy chains from the same G myeloma proteins differed from each other and from normal heavy chains. In general, the myeloma chains contained a larger number of well defined spots; some of these were common to normal heavy chains while others were unique to each protein. Others, present in normal heavy chains, were lacking in the myeloma proteins.

3. Comparison of the heavy chains and Fc fragments from the same protein suggests that much of the variability of different myeloma proteins and, presumably, antibodies resides in the Fd fragment.

4. Further support for this is given by the finding that the antigenic specificity of 3 myeloma proteins also appeared to reside in the Fd fragments.

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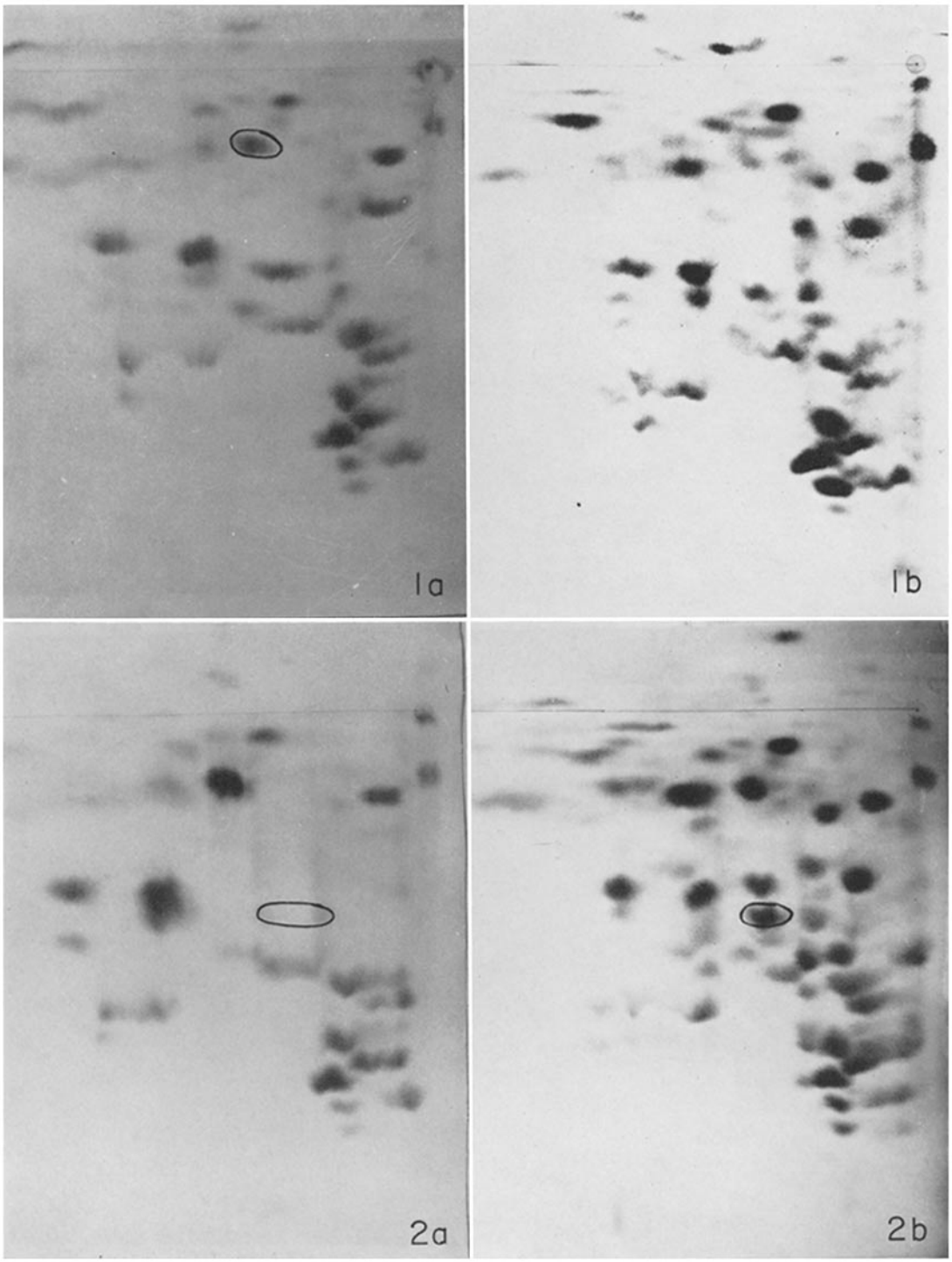
EXPLANATION OF PLATES

PLATE 1

FIGS. 1 *a* and 1 *b*. Peptide maps of Fc fragment of IgG from a normal Gm (a+b+f+) individual (Fig. 1 *a*) and γ-chain from a normal Gm (a−b+f+) individual (Fig. 1 *b*). The circle marks the Gm (a) spot.

FIGS. 2 *a* and 2 *b*. Peptide maps of Fc fragment (Fig. 2 *a*) and γ-chain from a Gm (a+) G myeloma (Ch) of type b (We) (Fig. 2 *b*). The peptide marked by circle is faint in the Fc fragment.

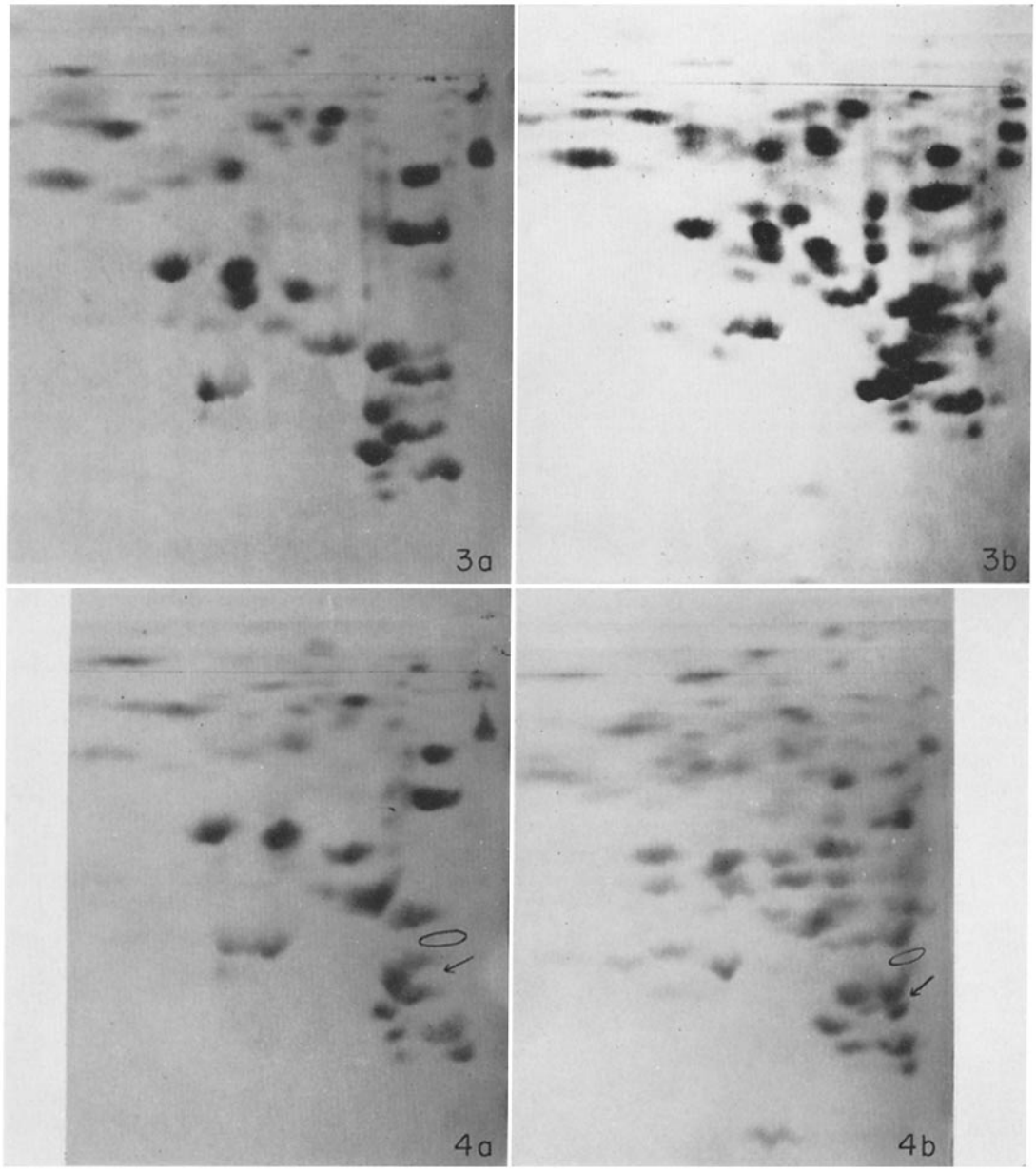


(Frangione and Franklin: Human immunoglobulins)

PLATE 2

FIGS. 3 *a* and 3 *b*. Peptide maps of Fc fragment (Fig. 3 *a*) and γ-chain from a Gm (f+) G myeloma (Pr) of type b (We) (Fig. 3 *b*).

FIGS. 4 *a* and 4 *b*. Peptide maps of Fc fragment (Fig. 4 *a*) and γ-chain of a Gm (b+) G myeloma (Gl) of type c (Vi) (Fig. 4 *b*). The arrow points to some of the peptide differences at the bottom. The circle marks the region of the missing peptide.



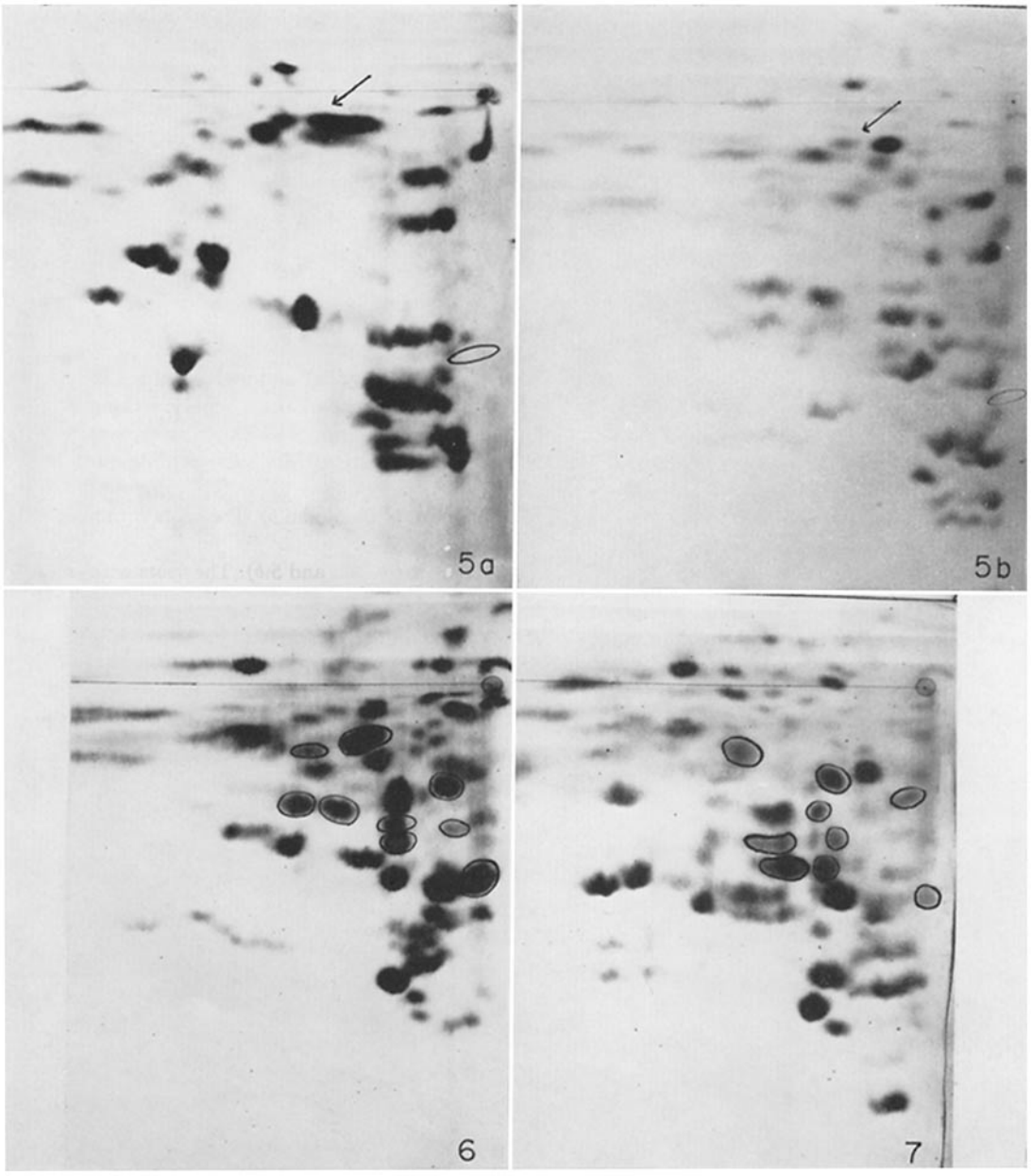
(Frangione and Franklin: Human immunoglobulins)

PLATE 3

FIGS. 5 *a* and 5 *b*. Peptide maps of Fc fragment (Fig. 5 *a*) and γ-chain of a Gm (b+) G myeloma (He) of type c (Vi) (Fig. 5 *b*). The circle marks a peptide lacking in the Fc fragment of this protein. The arrow points to 3 peptides which are prominent in about 20 per cent of normal IgG fractions. Here, too, there are some peptide differences at the bottom similar to those in Figs. 4 *a* and 4 *b*.

FIG. 6. Peptide map of the Fab fragment of Pr (Figs. 3 *a* and 3 *b*). The spots with a circle are contributed by the Fd fragments.

FIG. 7. Peptide map of the Fab fragment of He (Figs. 5 *a* and 5 *b*). The spots with a circle are contributed by the Fd fragments.



(Frangione and Franklin: Human immunoglobulins)