IMMUNOLOGICAL RELATIONSHIPS AMONG THE MYELOMA PROTEINS

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The unique occurrence of plasmacytoma and the frequently associated phenomena hyperproteinemia and Bence-Jones proteinuria in the disease, multiple myeloma, have provided the basis for many studies of their possible relationships. In no other disorder have there been encountered the changes in serum proteins which may occur in this disease. Although evidence is suggestive that the abnormal components arise from the neoplastic plasma cells (1–3) many questions concerning the synthesis of these proteins remain unanswered.

The extraordinary individual specificities of multiple myeloma have been demonstrated by physical studies of the myeloma serum proteins and urinary proteins (4–6). The abnormal serum component has been defined as a globulin which may range in electrophoretic mobility from that of a slow γ-globulin to that of an α-globulin (7). Hyperglobulinemia has been found to occur in approximately 62 per cent of the patients suffering from the illness (4).

Immunological studies of the Bence-Jones proteins (8, 9) have substantiated further the individual specificity of the disease from one person to another. Although the small sized urinary protein (molecular weight 24,000 to 90,000 (6)) may hypothetically be derived from myeloma serum proteins (molecular weight 120,000 to 200,000 (6)) there is no immunological evidence of this (8). On the basis of findings from isotopic studies Putnam (10) has suggested that the rates of synthesis of the Bence-Jones protein and serum myeloma protein are independent.

Wuhrmann, Wunderly, and Hässig (11) carried out the first studies defining immunological relationships among the abnormal globulin components in the sera of different patients. They concluded that the more rapidly migrating β-globulin type myeloma proteins were immunologically specific. This was not demonstrated in the case of the γ-globulin type myeloma proteins.

The immunological relationship existing between the abnormally elevated myeloma serum globulin and fractions of normal γ-globulin has been demonstrated in a previous study (12). The present report deals with a continuation of this work, in which the sera of 21 patients suffering from multiple myeloma were examined.

Methods and Materials

1. Myeloma Sera.—Most sera were hyperproteinemic, with the total protein levels ranging as high as 17 gm. per 100 ml. The electrophoretic mobilities of the abnormal myeloma

components were quite diverse, ranging from the mobility of a slow γ-globulin to that of β-globulin of intermediate mobility. On the basis of the mobility value, the myeloma proteins were classified as γ- or β-type, those of mobility less than 1.5 being designated γ-type and those of greater mobility, β-type. It is emphasized that this classification is a purely arbitrary division which serves best to describe the findings in this study. Throughout this report the

TABLE I

Patient	Serum total protein	Myeloma protein	Myeloma protein mobility protein	Sedimentation constant of myeloma protein	Relative reactivity of myeloma components with γ-G antiserum*
	gm./100 cc.	*gm./100 cc.*	$\times 10^{-5}$		
A	14.0	7.9	0.3 (R.)‡	6.4 (R)‡	3+
B	14.5	9.6	1.0		2+
C	12.1	8.1	1.0 (R.)‡	6.6 (R.)‡	3+
D			1.0		
E	13.9	8.4	1.1 (R.)‡	6.5 (R.)‡	3+
F	9.7	4.9	1.1		3+
G			1.0 (P.)§	6.5 (P.)§	
H	11.8	8.7	1.1 (C., P.)‖	7;10 (C., P.)‖	3+
I	5.6	3.0	1.2 (R.)‡	6.6 (R.)‡	tr
J	17.2	13.7	1.2		3+
K	13.4	9.2	1.9	6; 9	tr–1+
L	13.5	10.2	2.2		3+
M	8.9	6.4			3+
N	11.0	6.1	2.12	6; 9	tr–1+
O			2.3		tr–1+
P	12.5	6.1	2.3 (R.)‡	6.2 (R.)‡	f.tr
Q	9.8	5.2	2.5	6; 9	1+
R	9.5	5.2	2.3		tr–1+
S	12.0	7.2	2.8 (R.)‡	6	0
T	9.4	6.3	2.8		1+
U	9.8	7.1	3.0	6; 9	tr–1+

* Values indicated 1–4+ were estimated on the basis of 4+ reaction between normal γ-globulin and γ-globulin antiserum.

‡ R., data quoted from Rundles (6).

§ P., data quoted from Putnam (19).

‖ C., P., data quoted from Cogin and Peterman (17).

myeloma sera or proteins are represented by their alphabetical designation prefixed by the qualification of γ or β. The available data pertaining to this series are presented in Table I.

2. Normal γ-Globulin and Abnormal Myeloma Proteins.—Close immunological relationships have been shown to exist among different γ-globulin preparations (13–15). γ-Globulin fractions II-1,2, and II-3[1], prepared by ethanol fractionation and also fractions obtained by preparative zone electrophoresis (16) were used as normal standards. Dr. Geraldine Cogin and Dr. Mary Petermann of Sloan-Kettering Institute for Cancer Research, separated myeloma H protein from serum using the ethanol fractionation procedure and kindly made

[1] Obtained from E. R. Squibb and Sons, New York.

available the results of the physical studies (17). Electrophoretic analyses indicated that 98 per cent of the material remained a single component in solutions of ionic strength 0.1 between pH 4.0 and 10.0. In the ultracentrifuge it was shown to contain 95 per cent S = 7 component and 5 per cent S = 10 component. Myeloma Q, salted out with ammonium sulfate was electrophoretically one component.

The myeloma proteins P, K, and N were separated from serum by means of zone electrophoresis using the multiple paper strip preparative method (18), which made possible the separation of 3 to 4 ml. of serum in one run. Myeloma proteins A, E, S, and U were prepared by means of zone electrophoresis (16) in a starch supporting medium. In all the electrophoretic separations barbital buffer pH 8.6, μ = 0.1, was used. We are indebted to Dr. R. W. Rundles, Duke University Medical School, for the myeloma sera A, C, E, I, P, and S. The cryoglobulin type myeloma G was obtained through the kindness of Dr. Bernard Udin and Dr. Frank Putnam of the University of Chicago. It was obtained from the serum of Case 5 reported in their studies in 1953 (19); however, the sample provided had not been tested by them for homogeneity. Some of the data relative to these materials have been quoted from the authors' studies (6, 17, 19) and are indicated in Table I.

3. Immunological Procedures.—Rabbits weighing about 2 kg. were used for immunization. 20 to 30 mg. of protein antigen was injected per animal using the adjuvant technique described by Freund (20). This procedure was necessary because in previous studies considerable difficulty was encountered in developing antibodies to myeloma proteins. Antisera were developed for several preparations of normal γ-globulin as well as for purified myeloma proteins F, G, H, K, and Q. Six different antisera directed against preparations of normal γ-globulin were used to substantiate the findings in order that no conclusions be based on the results with one antiserum. Antibody titers reached satisfactory levels after 5 or 6 weeks. The rabbit sera were diluted to contain a titer of 150 to 200 μg. of antibody N per ml.

Precipitin studies were carried out using 0.05 or 0.10 ml. of antiserum, in which 15 to 20 μg. antigen N usually sufficed to develop an equivalence zone. The precipitates were brought to a constant dilution of 0.5 ml. with saline, allowed to stand at room temperature for 1 hour and then overnight at 4°C. The precipitates were washed four times with phosphate-saline solution buffered at pH 7.0. After each wash the supernatant fluid was sucked off leaving only 0.25 ml. in which to mix the precipitate preparatory to the next wash. After the final wash had been removed, all precipitates were evaporated to dryness in an evacuated desiccator.

Quantitative determination of the protein in the precipitates was carried out by means of a modification of the ninhydrin color method (21). Some precipitation in the colored solution was noted in the range of higher protein concentration when the reaction was carried out at pH 5.0. Adjustment of the reaction to pH 6.1–6.3 was effective in preventing the undesirable turbidity. A reference standard of normal γ-globulin was used to construct a quantitative standard curve. Precipitin studies were carried out, for the most part, in triplicate. It was possible to determine the nitrogen value of the precipitates within a range of 1 to 2 μg. N.

Agar diffusion studies were carried out according to the method of Ouchterlony (22). A modification (23) of the immunoelectrophoresis technique of Grabar and Williams (24) was used in which potato starch was utilized as the supporting medium for electrophoresis.

RESULTS

Physical Studies.—Electrophoretic analyses of the myeloma sera were done primarily by means of the filter paper method (25) and the mobility and concentration of each myeloma component calculated. Fig. 1 illustrates the pat-

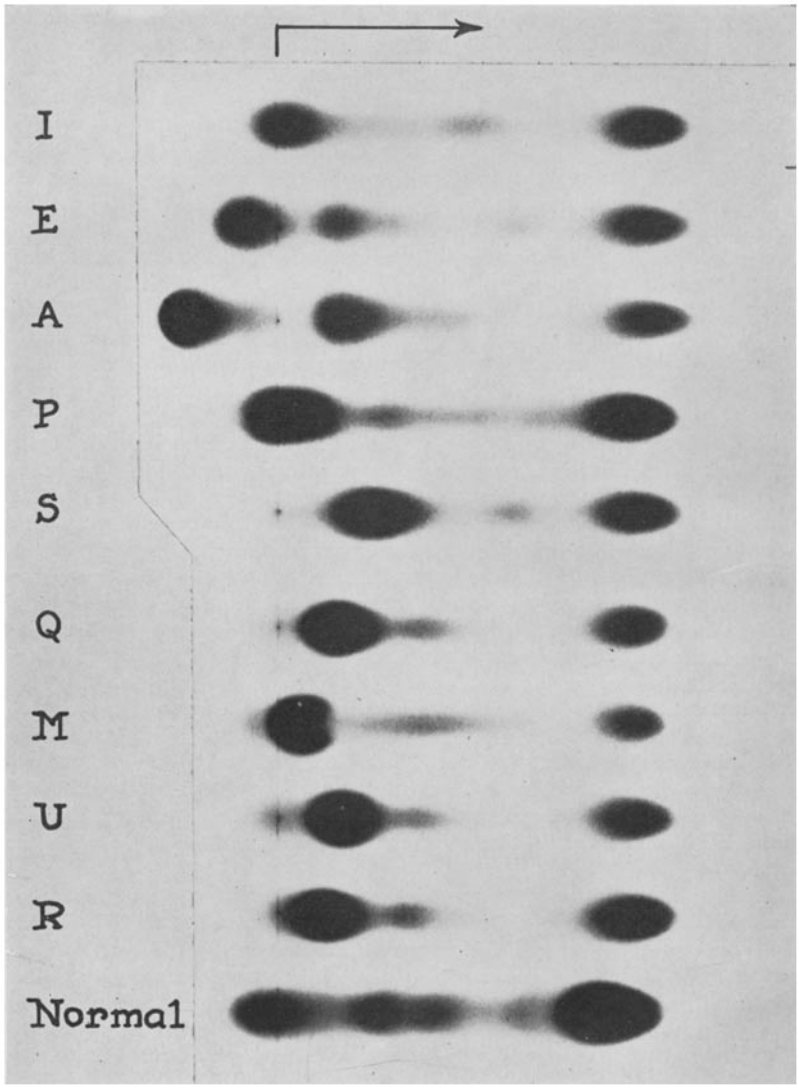

FIG. 1. The zone electrophoresis patterns on paper of 9 myeloma sera are shown in contrast with normal serum. The high concentration of myeloma proteins and decrease in other components are striking.

terns of nine myeloma sera and one normal serum produced on a single sheet of paper on which all sera were separated simultaneously under identical conditions. The great electrophoretic heterogeneity of the myeloma proteins in sera from different individuals is apparent, the mobility of these components rang-

ing from 0.3 to 3.0 in this study. Furthermore, Fig. 1 reveals the striking decrease in the other serum components at the expense of the tremendous increase in the pathological protein.

Ultracentrifugal analyses of certain of the purified myeloma proteins were carried out in the Spinco model E analytical machine. Three types of pattern were observed and are illustrated in Fig. 2. Analysis of the electrophoretically prepared normal γ-globulin which was used for immunization revealed a major component S=6.5 and a second component S=20 present in extremely small concentration. None of the S=10 component found in material prepared by

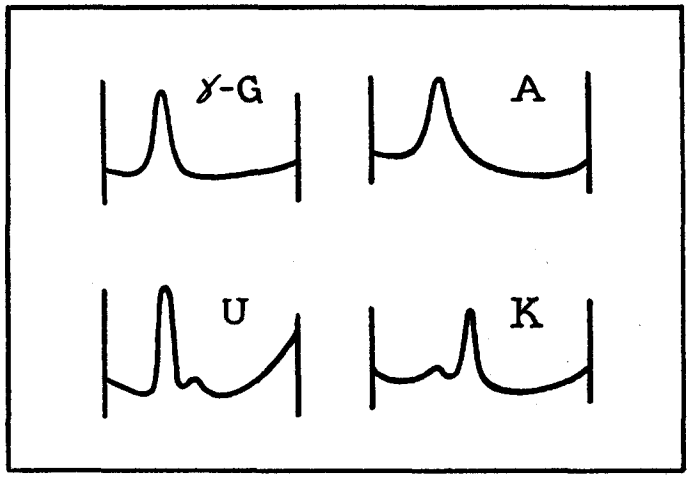

FIG. 2. The sedimentation patterns of three myeloma proteins and normal γ-globulin are shown. The variation in proportions of S = 6 and S = 9 components of U and K contrasts with the single S = 6 boundary found with myeloma A and normal γ-globulin.

ethanol precipitation was noted in γ-globulin prepared by means of electrophoretic separation. Certain of the myeloma sera consisted of only one major component similar to that of γ-globulin S=6 (Fig. 2, pattern A). In Rundles' studies (6) all the γ-myeloma proteins and the one β-myeloma protein reported sedimented as single moieties in the ultracentrifuge with values in the range S=6.5.

The second type of ultracentrifugal pattern consisted of a major S=6 and a minor S=9 component (Fig. 2, pattern U). The third type of pattern was made of a minor S=6 and a major S=9 component (Fig. 2, pattern K). These findings are similar to those of Putnam (19) in which certain of the β-myeloma proteins consisted of a major ultracentrifugal component S=9.5. It appeared that certain of the myeloma proteins differed in sedimentation as well as electrophoretic characteristics, a fact which further substantiated the individual specificity of this disease state.

Quantitative Precipitin Studies with Myeloma Sera.—A great variation in the degree of reactivity with antiserum to normal γ-globulin was noted when the myeloma sera were compared with normal serum. The myeloma sera reacted in much greater dilution than did normal serum. Furthermore, the intensity of the precipitin reaction in this region was quite variable but never as great as that of normal serum in its equivalence zone. Fig. 3 illustrates these marked differences for three myeloma sera compared with the reaction of normal serum. β-Myeloma serum L (mobility = 2.2) gave a more intense precipitin reaction than γ-myeloma serum B (mobility = 1.0). Both reacted with much greater intensity than a similar dilution of normal serum. However, β-myeloma serum

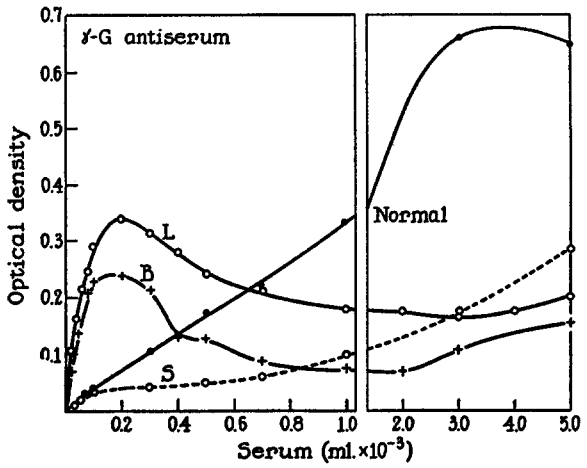

FIG. 3. The precipitin curves reflect the reactivity of three myeloma sera and a normal serum with antiserum to normal γ-globulin. The optical density refers to the ninhydrin color developed by the precipitates. None of the myeloma sera react with the intensity revealed by the normal serum.

S (mobility = 2.8) showed negligible reaction in comparison to myeloma sera B and L and also normal serum.

Although myeloma serum L reacted strongly with γ-globulin antiserum, this was not generally true of the β-myeloma sera. Most of these behaved in a manner similar to that of normal serum in great dilution, indicating very little reaction of the β-myeloma component with γ-globulin antiserum. The reverse was true of the γ-myeloma sera. The great majority of these reacted with antiserum at high dilutions in which it was apparent that the myeloma component was reacting with the antiserum. The intensity of the reaction at high dilutions was different for each myeloma serum.

The low order of reactivity revealed with myeloma S serum in Fig. 3 could not be accounted for by the myeloma S component. Studies with the purified

myeloma protein from this serum demonstrated a complete lack of reactivity with antiserum to normal γ-globulin. The reaction of the whole serum was based on the small amounts of γ-globulin present and not the myeloma protein. This was the only myeloma protein in the entire series which failed to react even to a slight degree with normal γ-globulin antiserum.

As increasing amounts of myeloma sera were added to γ-globulin antiserum the precipitin curves in Fig. 3 indicate a zone of antigen excess while normal serum was still rising through its prezone. Upon reaching the equivalence zone of normal serum, all the myeloma sera showed a variable increase in precipitating activity. It is possible that as more myeloma serum was added, the normal γ-globulin component which was present in decreased quantity became reactive presumably with that portion of the antiserum which did not form a complex with the abnormal myeloma protein.

These findings indicated that most of the myeloma sera contained an abnormal component in high concentration which reacted variably with antiserum to normal γ-globulin. The variation of the intensity of the reaction did not appear to be a function only of the concentration of the abnormal myeloma protein present in the serum. It was apparently related also to the extent of individual reactivity or closeness of immunological relationship of each myeloma protein to normal γ-globulin.

Quantitative Precipitin Studies of Purified Myeloma Proteins with Antiserum to Normal γ-Globulin.—The relationships among ten myeloma proteins and antiserum to γ-globulin were studied in order to clarify the findings with the myeloma sera. The high concentrations of these proteins in the sera made it possible to separate them in a high degree of purity.

Fig. 4 illustrates the data of the precipitin reactions of four myeloma proteins and normal γ-globulin with antiserum to normal γ-globulin prepared by means of electrophoresis. γ-Myeloma proteins F and H each reacted with approximately two-thirds the intensity of the homologous antigen. β-Myeloma protein N was repeatedly shown to develop a weak but well defined equivalence zone. β-Myeloma proteins K and Q likewise reacted with γ-globulin antiserum to a much lesser extent although the equivalence zones were not as sharply defined. These patterns of behavior were also shown to exist with the cryoglobulin γ-myeloma protein G (Fig. 5), with γ-myeloma proteins A and E (Fig. 8), and with β-myeloma protein U.

The results indicated that there was a relation between the intensity of the reaction and the type of myeloma protein, the γ-myeloma proteins reacting more intensely than the β-myeloma proteins. These relationships held true for antisera developed against different preparations of normal γ-globulin as well as antisera derived from different rabbits during the course of the study.

An approximate evaluation of the reactivity of all the myeloma proteins with antiserum to γ-globulin is depicted in the last column of Table I. In those cases

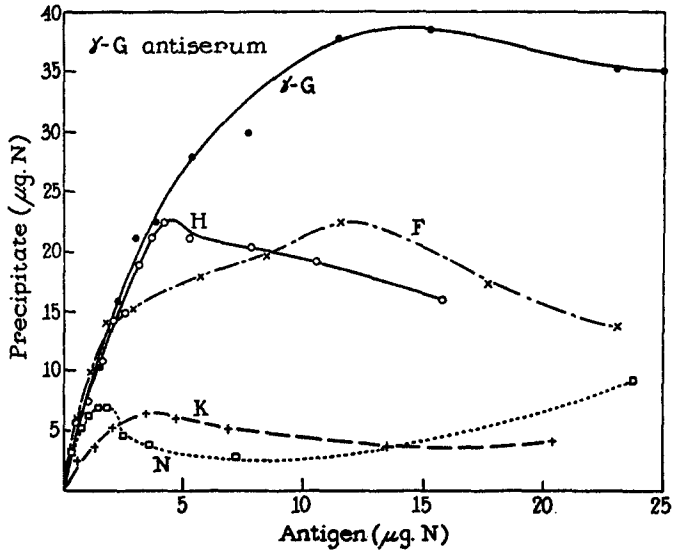

FIG. 4. The quantitative precipitin curves of four myeloma proteins compared with normal γ-globulin using antiserum to normal γ-globulin. Myeloma Q reaction, similar to that of K, has been omitted.

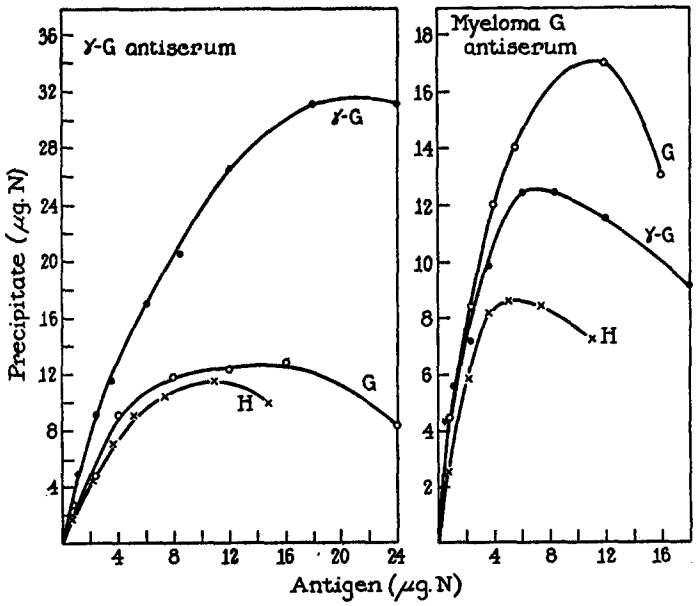

FIG. 5. The quantitative precipitin curves of two myeloma proteins G and H and normal γ-globulin are compared when antisera to γ-globulin (left) and myeloma G (right) are used. The specificity of each homologous system is apparent.

in which purified myeloma proteins were not prepared, an estimation of the myeloma protein reactivity could be derived from the intensity of reaction between the myeloma sera and γ-globulin antiserum. This quantitation is based on 4+ reaction for normal γ-globulin at its equivalence point as shown in Fig. 3. It was evident from the analysis of these proteins and also those of the previous study (12) that in no instance did they react as intensely with γ-globulin antiserum as did normal γ-globulin.

It appeared that the myeloma proteins would react with only a fraction of the antibodies directed against normal γ-globulin. These quantitative reactions substantiated the findings with myeloma sera (Fig. 3) that γ-myeloma proteins are immunologically related to γ-globulin. The reactivity of the purified fractions was similar to that of the corresponding whole sera. The β-myeloma proteins, reacting with variable and usually less intensity than the γ-myeloma proteins, appeared much more distantly related to γ-globulin.

Quantitative Precipitin Studies of Purified Myeloma Proteins with Antisera to Myeloma Proteins.—Antisera were developed against γ-myeloma proteins F, G, and H and β-myeloma proteins K and Q. The precipitin reactions are depicted in Figs. 5, 6, and 7. In each instance the myeloma protein reacted most intensely with its homologous antiserum. The reactivity of the heterologous antigens, although variable with respect to the different antisera, did show some order of relationship.

Antiserum to γ-type myeloma F reacted most intensely with its homologous antigen but did not distinguish as closely between normal γ-globulin, its homologous antigen and myeloma H as did the other antisera. No reaction occurred with β-myeloma protein K, N, or Q. (The lack of reaction is indicated by a straight line above the abscissa labelled K, Q in Fig. 6.) Conversely, antiserum to γ-type myeloma protein H differentiated readily the homologous reactivity from that of myeloma protein F and that of normal γ-globulin. The β-myeloma protein N gave a small but reproducible reaction as it did with γ-globulin antiserum. β-Myeloma proteins K and Q did not react with this antiserum. It appeared that γ-myeloma protein H had certain characteristics linking it to both γ-globulin and β-myeloma protein N.

A similar order of relations is found when the reactions of the proteins with the antisera to the β-myeloma proteins K and Q are considered as shown in Fig. 7. The β-myeloma proteins Q and N did not react as intensely as the homologous proteins with β-myeloma K antiserum. Of special interest was the complete lack of reaction between the γ-myeloma proteins F and H and this antiserum. However, a well defined reaction with γ-globulin was noted. β-Myeloma Q antiserum reacted not only with the other β-myeloma proteins but also with γ-myeloma H. Only γ-myeloma protein F failed to react with myeloma Q antiserum.

The cryoglobulin was γ-myeloma protein G. As in the other cases cited above

it showed only partial reactivity with γ-globulin antiserum when compared with homologous antigen. Likewise it reacted with its homologous antiserum

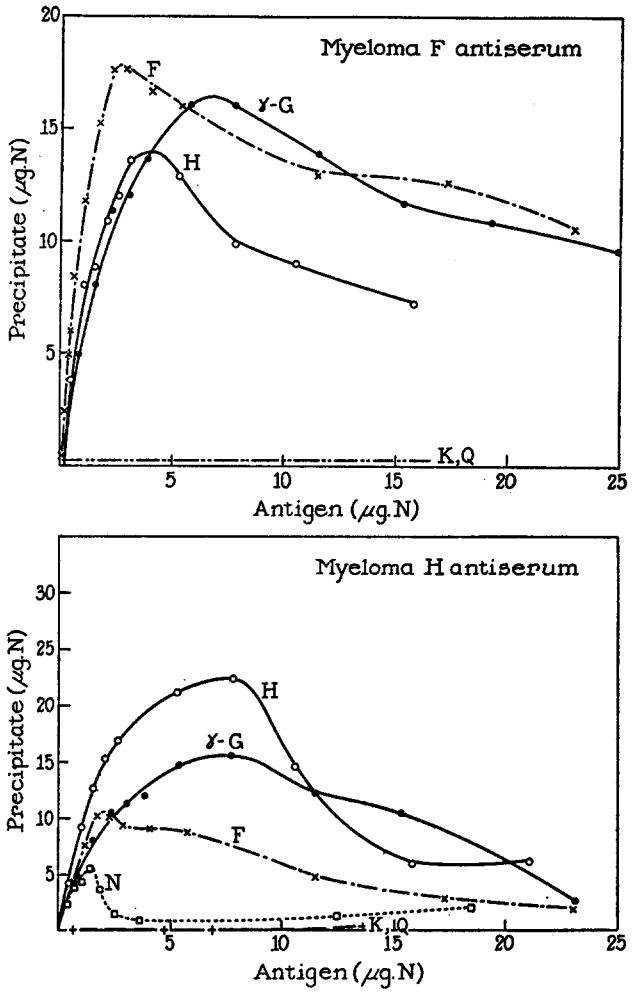

FIG. 6. The reactivity of antisera to two γ-myeloma proteins F and H with five myeloma proteins of γ-and β-type and normal γ-globulin are compared. The complete specificity of myeloma K and Q is denoted by the lack of reactivity charted as a zero line on the abscissa.

to a much greater degree than did γ-globulin or γ-myeloma H. The precipitin curves are shown in Fig. 5.

These precipitin responses illustrated clearly that the γ- and the β-myeloma groups of proteins were not as closely related as the individual proteins within each group were with each other. There did exist, however, certain immuno-

logical relationships which implied structural continuity between the groups of proteins despite the physical differences.

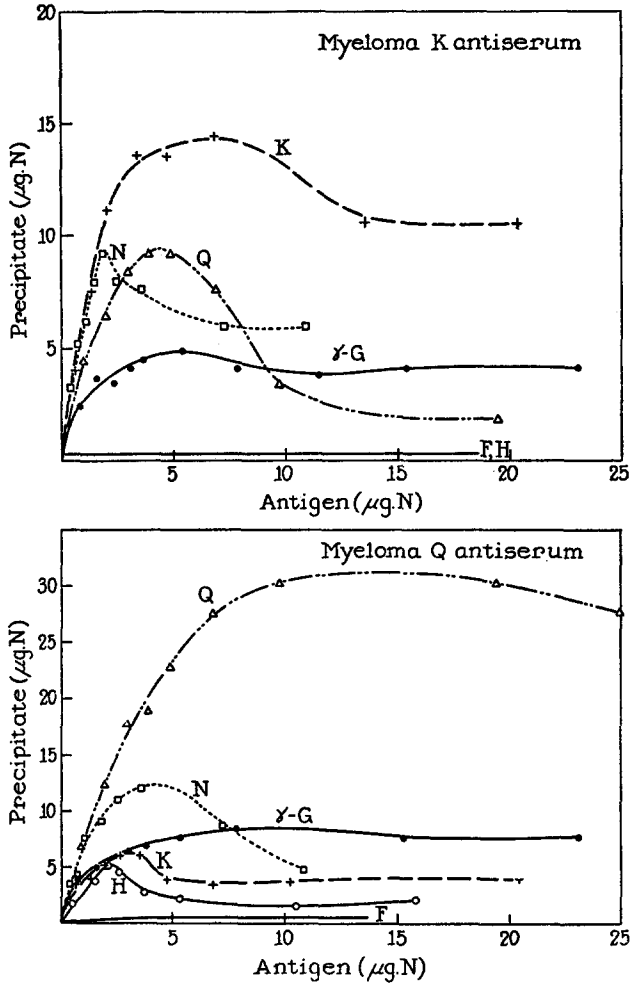

FIG. 7. The reactivity of antisera to two β-myeloma proteins K and Q are compared, as in Fig. 6.

Summary of Precipitin Reactions.—The intensity of the precipitin reactions in the foregoing experiments may be compared if the intensity of the homologous reactions are represented as 100 per cent. The results for five of the myeloma proteins and γ-globulin are tabulated in Table II. The fact that certain myeloma proteins failed to react with heterologous antisera indicated a high degree of specificity of the reactions. The data outside of the dotted lines

in Table II signify that a difference in behavior of the γ- and β-myeloma proteins is prominent when considered in terms of the intensity of the reactions with antiserum to γ-globulin. The γ-myeloma proteins precipitated a much greater percentage of γ-globulin antibody than did the β-myeloma proteins.

Similar findings were noted in γ-globulin reactivity with the antisera to myeloma proteins. It was apparent that there existed two differing orders of reactivity between the myeloma proteins and normal γ-globulin antiserum. The γ-type myeloma group appeared closely related to the normal γ-globulins. The β-type myeloma group was more distantly related and in one instance there was an absence of any immunological similarity to normal γ-globulin. However, the classification into γ- and β-types on the basis of electrophoretic mobility cannot be more than arbitrary. The immunological relationships do not observe such well defined parameters. Since β-myeloma L (mobility=2.2)

TABLE II

Antigen	Antibody precipitated from antiserum				
	γ-G	F	H	K	Q
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
γ-G	100	72	63	32	23
F	50	100	58	0	0
H	71	71	100	0	23
K	17	0	0	100	22
Q	28	0	0	60	100
N	25	0	33	76	45

reacted more intensely with γ-globulin antiserum than did γ-myeloma B (mobility=1.0) it is apparent that there must exist considerable overlapping of the two groups with respect to electrophoretic mobility.

The interrelationships of the myeloma proteins can be determined by a consideration of the data contained within the dotted lines of Table II. Antiserum to γ-myeloma F reacted only with F and H but not with K, Q, or N. γ-Myeloma H antiserum was reactive with F, H, and also N but not K or Q. β-Myeloma K antiserum reacted with K, Q, and N but not with γ-myeloma proteins F and H. It appears that in this particular system of proteins, β-myeloma N occupies an intermediary position, being related not only to β-myeloma K but also to γ-myeloma H. Since γ-myeloma F and H are immunologically related, it may be postulated that γ-myeloma F is related to β-myeloma K indirectly through the link of myeloma N. Similarly β-myeloma Q may be related to the other proteins because of the reaction of its antiserum with both γ-myeloma N and γ-myeloma H. The results with other myeloma proteins indicate that they may be fitted into a similar scheme.

These findings as well as the general relationship obtained with normal γ-globulin antisera suggest that while each of the myeloma proteins has an immunological specificity, each has characteristics which relate it directly or indirectly to the other proteins and to normal γ-globulin.

Relationship of the Myeloma Proteins to Normal γ-Globulin.—In attempting to interpret the results achieved with antiserum to the myeloma proteins, the question of immunological homogeneity of each system must be considered. Although the myeloma proteins were present in high concentration in the original sera, and undoubtedly represented a high percentage of the isolated protein material, the presence of other serum components must be assumed as minor impurities in those fractions separated by means of electrophoresis.

In order to demonstrate that such contamination did not explain the reactivity of the myeloma proteins with γ-globulin antisera the following experiments were carried out. β-Myeloma sera K, Q, S, and U were fractionated by means of zone electrophoresis in a starch supporting medium (16). Immunological quantitation of the amount of protein which would react with antiserum to normal γ-globulin of slow mobility was carried out for segments representative of the myeloma protein component as well as the γ-globulin component. The results have been recorded in detail elsewhere (23). Myeloma protein S failed to react with the γ-globulin antiserum. In K, Q, and U the amount of γ-globulin or γ-globulin–related protein in the myeloma protein component was found to be as much as 70 times that of the γ-globulin component. Although the myeloma protein in these instances was only weakly reactive with γ-globulin antiserum, the high concentration of the abnormal protein accounted for the reactivity of the myeloma serum in great dilution. Thus it was apparent that the major portion of material in the myeloma sera which was related to γ-globulin was located in the myeloma protein component.

Further studies were carried out to determine the effect of a myeloma protein containing known concentrations of added γ-globulin "impurity" upon the reactivity with γ-globulin antiserum. This seemed especially important in the case of the γ-type myeloma proteins in order to establish that the γ-globulin relationship was not based on incorporated γ-globulin in the test antigen. Fig. 8 illustrates the precipitin reactions of γ-globulin antiserum with homologous antigen γ-myeloma protein A; γ-myeloma protein A plus γ-globulin, ratio 1:1; γ-myeloma protein A plus γ-globulin, ratio 9:1. Again the limited but well defined equivalence zone of this myeloma protein A is apparent in the range of antibody excess relative to the homologous reaction. The quantitative precipitin curve resulting from the 1:1 mixture of myeloma A with γ-globulin illustrates the tendency of the added γ-globulin to mask the equivalence zone of the myeloma protein; however, two zones of reactivity can be seen. Likewise in the reaction with the 9:1 mixture of myeloma A and γ-globulin, the amount of γ-globulin "impurity" is insufficient to mask the

equivalence zone of the myeloma protein, but produces a well defined rise toward a second zone of reaction as increasing amounts of the antigen mixture are added.

These findings indicate that the immunological reactivity of the purified myeloma proteins cannot be explained on the basis of γ-gamma globulin impurity in the antigens.

Absorption Studies.—Previous investigations (12) revealed that serial absorption of γ-globulin antiserum with a γ-myeloma protein failed to remove all of the homologous reactivity. In the present study antisera to γ-globulin

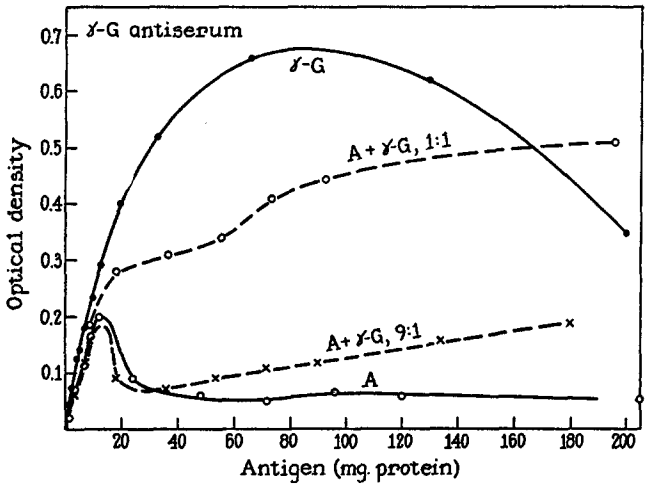

FIG. 8 The effects of mixing γ-globulin and purified myeloma A as test antigen are compared with the reactivity of the native proteins with antiserum to normal γ-globulin. The precipitates are recorded in optical density units produced by the ninhydrin reaction. γ-Myeloma E developed a precipitin reaction similar to that of γ-myeloma A.

and to the myeloma proteins were absorbed at a point corresponding to the individual equivalence zones. A second absorption using three times the amount of antigen at the equivalence point was also carried out. Table III records some of the results of these studies. Absorption of γ-globulin antiserum with γ-myeloma proteins F and H did not remove the homologous reactivity. Absorption with β-myeloma proteins K, N, and Q left reactivity for the homologous γ-globulin as well as for γ-myeloma proteins F and H. A similar picture prevailed in observations with antisera of the γ- and β-myeloma proteins, with the exception of γ-myeloma F antiserum. In this instance homologous reactivity was completely absorbed by the heterologous proteins, γ-globulin and γ-myeloma H, suggesting a close immunological relationship. However, the immunological difference between these two was clear when the reverse ex-

TABLE III

Antigen	γ-G antiserum absorbed with					
	γ-G	F	H	N	K	Q
γ-G	0	2+	2+	4+	4+	4+
F	0	0	0	4+	3+	3+
H	0	0	0	2+	3+	3+
N	0	0	0	0	+	+
Q	0	0	0	+	tr	0
K	0	0	0	+	0	+

Antigen	H antiserum absorbed with					
	γ-G	F	H	N		
γ-G	0	tr	0	3+		
F	0	0	0	2+		
H	+	2+	0	3+		
N	0	0	0	0		
Q	0	0	0	0		
K	0	0	0	0		

Antigen	K antiserum absorbed with					
	γ-G			N	K	Q
γ-G	0			0	0	0
F				0	0	0
H					0	0
N	2+			0	0	0
Q	2+			0	0	0
K	4+			2+	0	3+

Antigen	Q antiserum absorbed with					
	γ-G			N	K	Q
γ-G	0			tr	+	0
F						
H						
N	2+			0	0	0
Q	4+			3+	2+	0
K	2+			0	0	0

Antigen	G antiserum absorbed with					
	γ-G	H	G			
γ-G	0	0	0			
H	0	0	0			
G	+	+	0			

periment was carried out. In this case H antiserum could only be partially absorbed with γ-myeloma F leaving specific reactivity for the homologous protein as well as for γ-globulin. β-Myeloma protein reactivity was removed much more readily than that of γ-myeloma proteins in the γ-myeloma antisera studies and *vice versa*.

In certain instances (γ-globulin, myeloma G and H) exhaustive absorption of antisera was carried out, antigen being added far into the antigen excess zone by serial additions over a period of 5 days. Homologous reactivity could not be removed by heterologous antigens, although the intensity of the homologous reaction in each case was markedly diminished.

The findings of the absorption studies add weight to the concept that each of the myeloma proteins has an immunologically distinctive property which makes it different from every other protein. The pattern of behavior is similar to that characteristic of cross reactions.

Agar Diffusion Studies.—The interrelationships amongst the myeloma proteins and normal γ-globulin could also be demonstrated by means of the agar diffusion technique described by Ouchterlony (22). The high degree of purity of the reacting systems was emphasized by the single precipitin lines revealed in the agar diffusion analysis of the homologous systems. This held true for antiserum prepared against electrophoretically separated normal γ-globulin and fraction II-1, 2, and for myeloma proteins H, F, K, and Q. Antisera to myeloma G, however, revealed more than one antigenic component in the purified antigen.

The differentiation between immunologically related and distinct proteins was readily ascertained. Fig. 9 illustrates four different reactions defined by this method in the present study. In Fig. 9*a* a mixture of human albumin and γ-globulin antisera diffused from chamber 1 (top) towards the antigen serum albumin in chamber 2 and γ-globulin in chamber 3. The sharply demarcated crossing of the precipitin lines indicates the complete lack of immunological relationship. However, in Fig. 9*b*, γ-globulin was used as the antigen in both chambers 2 and 3 and the homologous antiserum placed in chamber 1. The union of the two precipitin lines indicates the immunological identity of the reactants in contrast to the results in Fig. 9 *a*. The quantitative precipitin studies revealed complete immunological heterogeneity between certain of the myeloma proteins. Such was the case between γ-myeloma H and β-myeloma K as well as γ-myeloma F and β-myeloma K and Q. Fig. 9*c* illustrates these findings. Antiserum to β-myeloma K diffused from chamber 1 against γ-myeloma H in chamber 2 and the homologous antigen, myeloma K in chamber 3. The distinct sharp homologous reaction stands in striking contrast to complete lack of heterologous reaction. Exactly similar results were obtained when the reverse of this system was studied; *i.e.*, γ-myeloma H antiserum *versus* β-myeloma K and homologous antigen.

An example of the results obtained when the interreacting myeloma proteins were studied in this manner is shown in Fig. 9*d*. Antiserum to γ-globulin diffused from chamber 1 against homologous γ-globulin in chamber 2 and β-myeloma protein Q in chamber 3. The homologous reaction formed a distinct band. The heterologous reaction likewise formed a distinct precipitin line, however, it united smoothly with the homologous γ-globulin precipitin line. The well defined spur of the homologous precipitin line extending beyond the point of

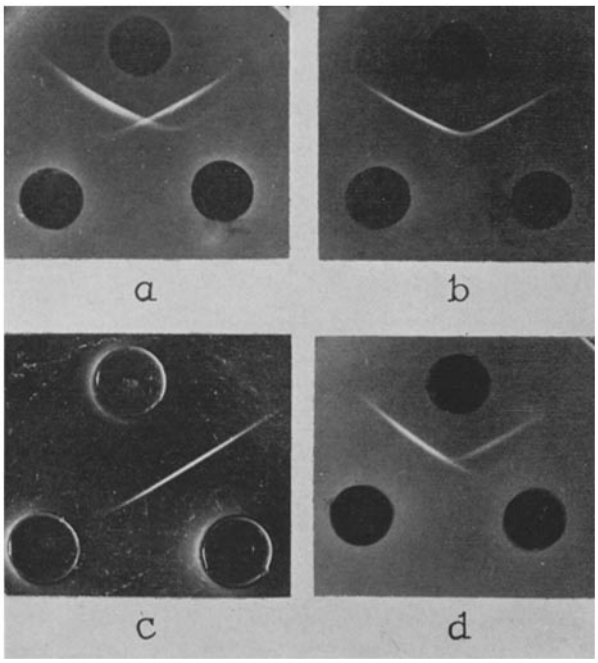

FIG. 9. The agar diffusion reactions illustrate (*a*) complete immunological specificity, (*b*) complete immunological similarity, (*c*) lack of immunological cross relationship, (*d*) cross-reacting immunological relationship. For explanation, see text.

union of the two precipitin lines and the complete absence of crossing of the lines are characteristic of the findings with cross-reacting proteins. A second faint band can be seen parallel to the heterologous reaction and continuous with the end of the spur of the homologous reaction. This is likely caused by a small amount of normal γ-globulin present as a contaminant in this preparation of myeloma protein Q. Similar results were obtained with the other interreacting γ- and β-myeloma proteins.

It was possible to substantiate these findings further by means of the immunoelectrophoresis technique of Grabar and Williams (24). This was modified by the use of starch as the supporting medium for the electrophoretic

separation. Normal serum and myeloma sera H, K, Q, and U were subjected to analysis. When antiserum to γ-globulin was used, the continuity of the elliptical myeloma protein precipitin line with the asymmetric precipitin line

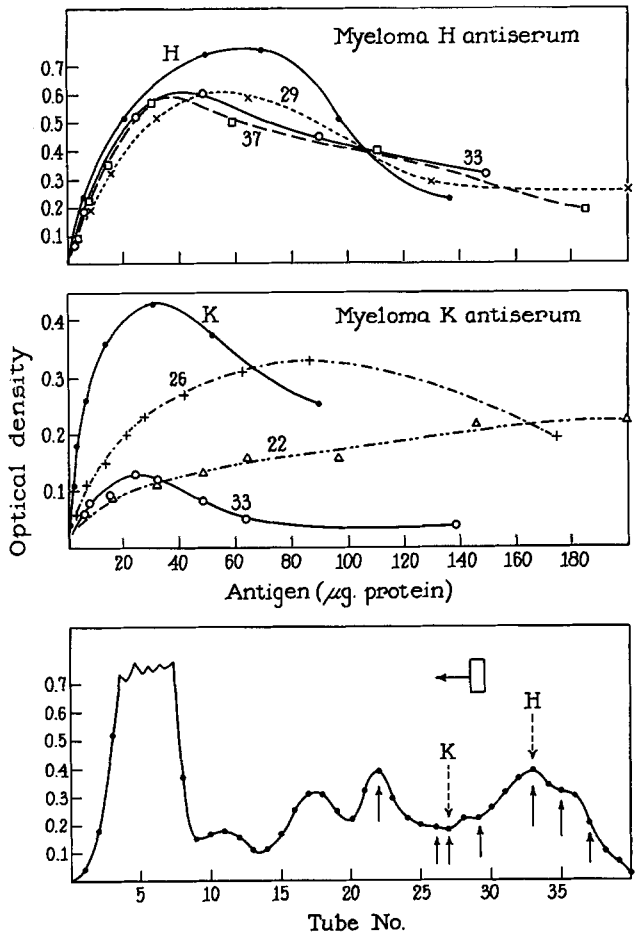

FIG. 10. The lowest pattern illustrates the zone electrophoretic separation of normal serum. Origin and direction of migration indicated by box and arrow. Broken arrows indicate mobility of two of the myeloma proteins whose antisera were tested. Solid arrows indicate fractions of normal serum used for testing. The upper two patterns demonstrate curves of quantitative precipitin reactions between numbered fractions and the myeloma antisera.

produced by the normal γ-globulin component of the sera in each case was in agreement with the findings of the Ouchterlony method outlined above. The evidence from these studies favored the concept of interrelationships of the myeloma proteins to each other and to normal γ-globulin on the basis of cross reactivity.

Differentiation of Normal Serum Components by Means of Myeloma Antisera. —In view of the relationships among the myeloma proteins and normal γ-globulin components studies were carried out to define the reactions between fractions of normal serum and antisera to the myeloma proteins. Normal serum preparations of varying mobility were obtained from a zone electrophoresis separation in a starch supporting medium. Antisera to γ-myeloma H (mobility=1.1), β-myeloma K (mobility=1.9), and β-myeloma Q (mobility=2.5) were used. These antisera were immunologically specific and homogeneous by the criterion of agar diffusion analysis.

The resultant precipitin reactions are presented in Fig. 10. The lowest section illustrates the electrophoretic pattern of normal serum from which test antigens were derived through the γ- and β-globulin regions, as indicated by the tube numbers. The reactions between γ-myeloma H antiserum and the test antigens are depicted in the uppermost section of Fig. 10. As in the previous experiments none of these antigens reacted with the same intensity as did the homologous protein, myeloma H. However, each of the fractions which included tubes 27, 29, 33, 35, and 37 reacted with approximately the same degree of intensity, using as the criterion the height of the equivalence zone in each case. Even certain fractions migrating in the β-globulin region appeared closely related to those in the main γ-globulin peak. This broad distribution of γ-myeloma related protein confirms previous evidence of a broad distribution of γ-globulin obtained with antiserum to γ-globulin of slow mobility (23).

The reactions between β-myeloma K antiserum and the normal serum fraction were in marked contrast to the above findings and are depicted in the center section of Fig. 10. Again none of the antigens reacted with as great intensity as did the homologous protein myeloma K. The striking observation in this experiment was the extent of immunological reactivity of a fraction migrating faster than the main γ-globulin peak. The normal serum fraction of mobility similar to that of the original myeloma protein K, tube 27, reacted more intensely than did fractions of more divergent mobility. In agreement with the previous findings a distinct but minimal degree of reactivity was found between the main γ-globulin component and the β-myeloma antiserum. β-Myeloma Q, however, did not show this specific relationship to normal β-globulin components.

It was apparent that the immunological reactions of the myeloma antisera were not based on inhomogeneity of the antiserum. If that were the case a single band formed with the homologous antigen by the Ouchterlony agar diffusion procedure would not be expected in the presence of the strong reactivity given by the normal serum component of mobility similar to that of the homologous antigen. Agar diffusion analysis of these reactions revealed that the precipitin bands formed by the normal serum components behaved in a manner suggestive of cross reactions according to the criteria outlined above.

Since there exists a close immunological relationship among at least a por-

tion of the normal γ-globulins distributed throughout the electrophoretically defined γ- and β-globulin regions, an additional normal serum fraction related to certain β-myeloma proteins must be postulated. This component would appear to have properties which differ from the main γ-globulin group as well as the major β-globulin group of proteins.

DISCUSSION

The precipitin reactions described in this study indicated that every one of the myeloma proteins was different and individually specific. It appeared that the γ-myeloma proteins were all related to normal γ-globulin or a fraction thereof and, although different in each patient, all were related to one another. The β-myeloma proteins likewise were interrelated despite physical differences, and formed a distinct group having unique specificities. The well defined but limited reactivity of most of this group of β-myeloma proteins with γ-globulin antiserum could not be explained on the basis of antigenic impurity.

This study dealt with proteins ranging in electrophoretic mobility from 0.3 to 3.0 and which in the ultracentrifuge revealed the presence of single (S=6.5) as well as double (S=6.5 and 9.5) components. These physical differences have been amplified by the studies of others. Despite differences in the proportions of certain amino acids, Grisolia and Cohen (29) have defined the over-all similarity in amino acid composition of myeloma proteins and γ-globulin proteins. Smith (26, 27) and McFadden (28) have shown a fundamental similarity in amino acid composition of γ-globulin fractions; however, sufficient variation in the free amino groups was noted to make chemical differentiation possible. Likewise the variation in terminal amino acids described by Putnam (10) suggests a basis for the differences noted in the mobility of myeloma proteins. It is apparent that the myeloma proteins in common with normal γ-globulins differ in part by the number of free ionizable groups. Recent immunological studies (23) have revealed a wider distribution of γ-globulin related proteins throughout the spectrum of normal serum globulin components than are defined by electrophoretic separation. It is notable that the range and frequency of distribution of myeloma proteins correspond in general to those of normal γ-globulins, a point emphasized by Putnam and Udin (19).

On the basis of the reactions of normal serum fractions prepared by electrophoresis with β-myeloma antisera it was possible to differentiate at least a portion of the γ-globulin related material in the β-globulin region from γ-globulins of slow mobility. Certain components of the γ- and β-globulins are closely related to slow migrating γ-globulin. However, some of the fractions of fast migrating γ-globulin and β-globulin were shown by means of the β-myeloma antisera to have marked specificities of their own although distantly related to slow migrating γ-globulin. These findings substantiate the conclusion that despite the direct or indirect relationships existent among all the myeloma

proteins, the β-myeloma proteins comprised a group quite distinct from the γ-myeloma proteins. Instead of having a major relationship to the broadly distributed γ-globulin proteins, certain of the β-myeloma proteins appeared to be more closely related to another normal serum fraction located primarily in the region between the main γ-globulin and β-globulin peaks and extending into the β-globulin area.

It was clear that no hard and fast division between the two groups could be defined on the basis of electrophoretic mobility. An arbitrary classification was used in this study for the purposes of description. Considerable overlapping must in fact occur since there were noted certain fast migrating β-type myeloma proteins highly reactive with γ-globulin antiserum as well as γ-myeloma proteins of much less intensive reactivity whose mobility was similar to that of some of the strong reactors. As a general rule, however, the classification into two broad groups, γ- and β-type myeloma proteins, was valid when considered in the light of both electrophoretic and immunological characteristics.

Cohn's (15) immunological study of four fractions of normal γ-globulin indicated that the relative amounts of common antigenic components were different in each of the four preparations. In the present work a somewhat different order of relationships has been shown to hold for the reactions between myeloma proteins and γ-globulin. It was found in several instances that two myeloma proteins could have complete immunological specificity and yet both be related to normal γ-globulin. Such was the case with myeloma H and K as well as myeloma F and Q. The most simple operational system to which such reactions can be reduced may be derived from a consideration of the first case. If γ-globulin be considered to consist of three distinct determinants X, H, and K, it is apparent that the myeloma constituent H will be completely heterologous with respect to myeloma K and that absorption of γ-globulin by either will fail to remove reactivity of the other. Furthermore, the γ-globulin contains a completely specific determinant, X, which is unrelated to the myeloma groups. Such an explanation is borne out by the precipitation and adsorption studies presented above.

The question whether the relationships exhibited by these proteins are based on a system of true cross reactions or are due to antigenic impurities has been considered in this study. Landsteiner (30) noted that antibodies are severally directed toward distinctive groupings of the antigen. A repetition of certain amino acid patterns and gradual variation in determinants as might be expected in this series of proteins, based on physical similarities, could be operative in determining, at least in part, the observed variation in reactivity.

There is no evidence available which demonstrates that γ-globulin components which have thus far been purified, obey the laws of single antigenic systems. In order to demonstrate more than one immunologically active component in either antigen or antiserum it is necessary to carry the precipitation

reaction far into the range of antigen excess. In this study the equivalence zones of the myeloma proteins occurred in the range of antibody excess relative to the homologous reaction (with the exception of γ-myeloma F which was shown to be closely related to γ-globulin). This is to be expected in a cross-reacting system and has been shown for egg white proteins by Wetter (31) and serum albumins of different species by Melcher (32) and others (33, 34). Further evidence favoring cross reactions was that the most intense reactions were obtained with the homologous antigen in each instance. With the exception of antiserum to myeloma F, exhaustive absorption did not remove the homologous reactivity. The specificity of these systems was defined further by the complete lack of reactivity between certain of the myeloma proteins and heterologous myeloma antisera. The above evidence and that derived from the agar diffusion studies greatly favored cross reactivity as the basis of the interrelationships amongst these proteins.

Insufficient knowledge of the properties of normal serum proteins has made it difficult to determine conclusively whether multiple myeloma proteins are truly abnormal entities or simply represent a great elevation of a single one of the many normal globulin components. In general the data obtained in the present study favor the hypothesis that these proteins are not normal but related to normal constituents. The finding that in no instance did a fraction of normal serum react with myeloma protein antiserum as intensively as the homologous protein as well as the special property of some of the myeloma proteins to behave as cryoglobulins although closely related to normal γ-globulin favors this view.

It is tempting to consider the myeloma proteins as intermediates in the synthesis of γ-globulin and antibodies with the more closely related myeloma proteins having most of the determinant groups of γ-globulin. However, definite evidence on this point is lacking. The molecular size of the myeloma proteins does not suggest intermediate components unless the low molecular weight Bence-Jones proteins fit this category. The latter, however, have been shown to be immunologically distinct from both the myeloma proteins and normal γ-globulin (8).

The production of such a spectrum of abnormal proteins in large quantity is of interest because there is recognized histologically a wide variation in plasma cell morphology from one patient to another. The finding that every one of the myeloma proteins was different and individually specific is especially interesting in view of the belief that the proteins arise from the plasma cell which has undergone malignant hyperplasia. As these proteins might thus be considered by-products of the cancerous cells, it is possible that in other neoplastic disorders there may occur individually specific metabolites which qualify the disease from one person to another.

SUMMARY

An immunological study of 21 myeloma sera was carried out to determine their relationship to components of normal γ-globulin and to each other. Ten proteins were separated for detailed characterization.

Every one of the myeloma proteins studied was immunologically different, indicating individual specificity.

The γ-type myeloma proteins were all related to normal γ-globulin or a fraction thereof, and although each was distinct from the others, all were related.

The β-type myeloma proteins likewise were related to one another, though individually specific.

Evidence was found favoring the view that some of the γ-type myeloma proteins are related to the β-type myeloma proteins although most of them were completely unrelated. Likewise the results indicated a definite but distant relationship between the β-myeloma proteins and normal γ-globulin.

Certain of the β-myeloma proteins were more closely related to a fraction of normal serum of similar electrophoretic mobility.

It is suggested that most multiple myeloma proteins fall into two groups or families of cross-reacting abnormal proteins, one closely related to normal γ-globulin, the other distantly related to γ-globulin with marked individual specificities.

The authors are indebted to Dr. Melvin Cohn and Dr. Elvin Kabat for their valuable discussion during the course of the study.

Addendum.—Lohss and associates (35, 36) have recently described similar immunological studies employing γ-globulin and myeloma antisera. Their conclusions regarding the specificity of the myeloma proteins and variable relationship to normal γ-globulin correspond to those of the present paper.

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