RECOMBINATION OF HEAVY AND LIGHT CHAINS OF HUMAN γA–MYELOMA PROTEINS: FORMATION OF HYBRID MOLECULES AND CONFIGURATIONAL SPECIFICITY*

By ROBERT A. PRENDERGAST,‡ M.D., HOWARD M. GREY,§ M.D., and HENRY G. KUNKEL, M.D.

(*From The Rockefeller University*)

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A number of studies have demonstrated that the isolated heavy and light chains of γG-globulin reassociate readily to form four chain 7S γ-globulin molecules (1, 2). Recently it has been found that considerable specificity is involved in this interaction when homologous chains from myeloma proteins (3) or isolated antibodies (4) are placed in competition with heterologous chains. No studies are available concerning γA- and γM-globulin which represent the other major classes of immune globulins. It might be expected that the light chains from these other classes would behave similarly to those of γG-globulin since they are under similar genetic control (5). The heavy chains, however, differ markedly between each class (6).

Initial efforts at isolation and reassociation of γA– and γM–globulin chains indicated that under the conditions studied the heavy chains of γA-globulin reassociated with different light chains considerably more readily than those of γM-globulin. As a result, primary effort was directed toward the γA-globulin system. Mixed molecules of γA- and γG-globulin were readily formed, which involved both the kappa and lambda[1] light chains of either species. Antigenic studies of the reformed molecules containing γA–heavy chains revealed some interesting effects of quaternary structure on antigenic specificity.

Materials and Methods

Separation of Myeloma Proteins.—Sera were obtained from patients with electrophoretically and immunochemically confirmed diagnoses of multiple myeloma of either γA- or γG-class.

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‡ Present address: The Wilmer Ophthalmological Institute, The Johns Hopkins University School of Medicine, Baltimore.

§ Present address: The Scripps Clinic and Research Foundation, La Jolla, California.

[1] The nomenclature adapted by the World Health Organization has been utilized in this report. To avoid confusion the terms kappa (κ) and lambda (λ) have been utilized throughout for group I and group II myeloma proteins as well as light chains.

Myeloma proteins were isolated by starch block electrophoresis (7), concentrated by negative pressure vacuum dialysis, and protein concentrations determined by the Folin method (8). Immunoelectrophoresis and Ouchterlony double diffusion in agar were performed to establish the electrophoretic mobility, immunoglobulin class, and L chain group of each protein.

Preparation of L and H Chains.—Reductive cleavage of myeloma proteins was carried out by the method of Fleischman, Pain, and Porter (9). Following concentration to 20 to 30 mg per ml the protein was dialyzed 12 hr against 0.55 M tris buffer pH 8.5. This material was then placed in a nitrogen atmosphere and rendered 0.2 M with respect to mercaptoethanol. After 1 hr sodium iodoacetate was added to a final concentration of 0.3 M and the mixture stirred for 1 hr, followed by dialysis against 1.0 M propionic acid for 12 hr. The reduced and alkylated chains were then placed on a 3 × 70 cm Sephadex G-100 column equilibrated with 1.0 M propionic acid and eluted with this material at a flow rate of approximately 10 ml per hour at 4°C. Analysis of the protein concentration of various fractions by Folin technique showed clear separation into 2 peaks representing H and L chains. Fractions under each peak were pooled individually, dialyzed against several changes of distilled water, and brought to neutrality by final dialysis against 0.005 M tris buffer over a period of 72 hr at 4°C. The separated chains were then concentrated by vacuum dialysis to a final protein concentration of 4 to 10 mg per ml. In the case of the L chain pool 200 mm of mercury negative pressure was maintained through the concentrating step. Relative purity of the isolated chains was determined by Ouchterlony double diffusion analysis using appropriate specific antisera to detect L chain group determinants in the concentrated fractions from both peaks. In addition, immunoelectrophoresis was carried out to show electrophoretic homogeneity of each of these fractions.

Recombination of H and L Chains.—Aliquots of L chains were labeled with either I^{125} or I^{131} according to the method of Helmkamp et al. (10) with 1 mole of iodine per mole of protein. Recombination was effected by placing H and L chains in various molar ratios ranging from 0.5 to 1.5 in collodion membranes followed by dialysis against 0.1 M propionic acid, 2 changes of distilled water, and finally, 0.005 M tris buffer to neutrality over a period of 48 hr. Linear sucrose density ultracentrifugation was employed using a 5 to 20% sucrose gradient in 0.01 M tris-hydrochloride buffer pH 8. Following ultracentrifugation over a period of 12 to 16 hr, fractions were counted for radioactivity of I^{125} and I^{131}. The degree of radioactivity of each fraction was plotted against the per cent of gradient volume, 100% representing 5.0 ml and the top of the gradient (see Fig. 1). The relative position of both free L chains and the recombined 7S molecule was quite reproducible. Trace amounts of bacterial alkaline phosphatase with a sedimentation coefficient of 6.1S (uncorrected) were incorporated in the material placed on density gradients as a marker, the position of which could be determined by reaction of aliquots of ultracentrifugation fractions with appropriate substrate.

Effectiveness of recombination was also assessed by the relative electrophoretic mobility of L chains when in the free and combined state as determined by immunoelectrophoresis using specific anti-L chain antisera.

Antisera.—Antisera were prepared in rabbits to γA- and γG-myeloma proteins and various Bence Jones proteins. In addition, Cynomologous monkeys were immunized with γA-myeloma proteins and isolated γA-H chains.

RESULTS

Isolation and Recombination of H and L Chains.—Following Sephadex G-100 gel filtration of reduced and alkylated γA-myeloma proteins, analysis of the various fractions showed clear separation into two peaks as determined by Folin analysis. The first peak consisted of H chains, although in the case of kappa proteins varying degrees of contamination with unreduced or partially reduced

material were often noted. In contrast, similarly processed lambda proteins were shown to contain no L chain contaminants on Ouchterlony and immunoelectrophoretic analysis of material in the H chain peak. Subfractionation of this peak into several portions depending on their elution time did not markedly improve the immunochemical purity of the H chains of kappa molecules. The L chain

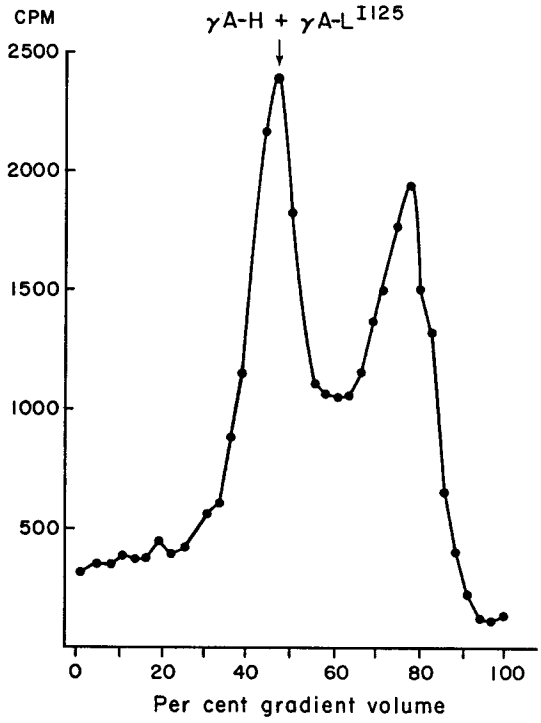
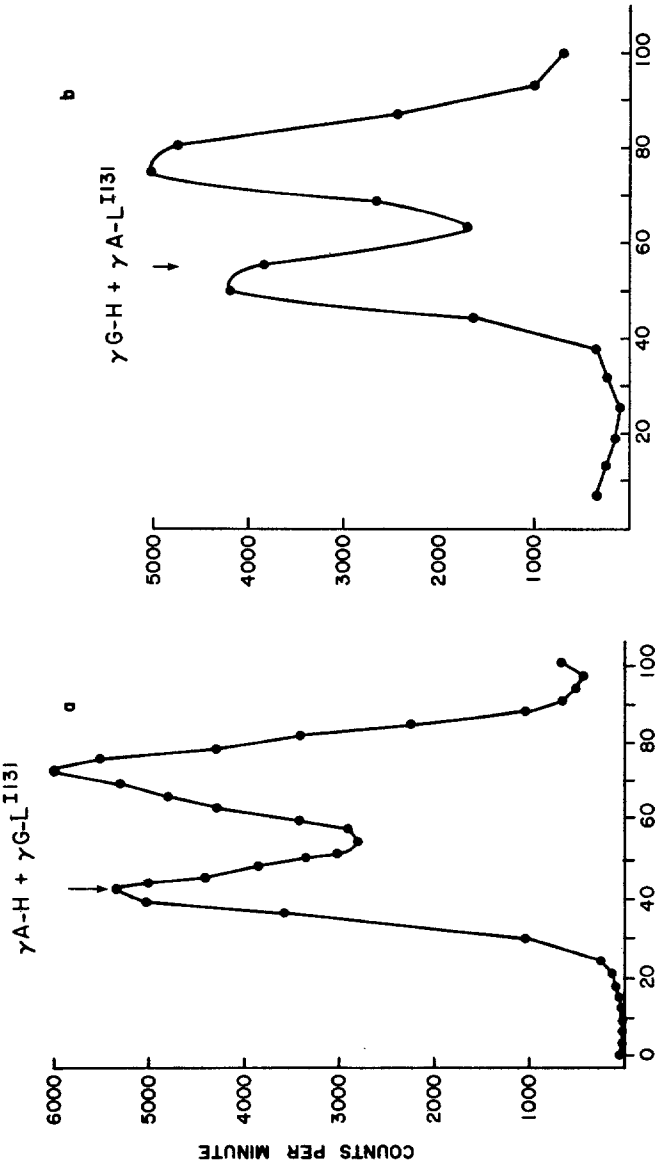


FIG. 1. Sucrose density gradient ultracentrifugation pattern illustrating mobility of labeled L chains in homologous recombined L and H chains in the lower, and residual free L chains in the upper portion of the gradient. Gradient of 5 to 20% sucrose from right to left was employed in this and subsequent patterns. The arrow represents the fraction showing the highest enzyme activity of the alkaline phosphatase marker.

peak of both kappa and lambda proteins was electrophoretically homogeneous and contained no heavy chain determinants by Ouchterlony double diffusion analysis.

Linear sucrose density gradient analysis of isolated labeled L chains from γA–myeloma proteins showed a sharp homogeneous zone of high specific activity present in the upper portion of the gradient at approximately 80% of the gradient volume, widely separated from the position of the alkaline phosphatase marker. When L chains were reacted with H chains from the same parent mye-



FIGS. 2 *a* and 2 *b*. Sucrose density gradient ultracentrifugation patterns illustrating hybrid molecule formation. Fig. 2 *a*. Pattern obtained with hybrid molecule composed of γA-H–γG-L chains. Fig. 2 *b*. Pattern obtained with hybrid molecule composed of γG-H–γA-L chains.

loma protein in a molar ratio of 0.8 to 1, significant recombination was seen to take place as shown in Fig. 1. Two clearly separated peaks of high specific activity are present, the first in the region of the alkaline phosphatase marker representing the recombined molecule and the second in the upper portion of the gradient in the region identical to that of free L chains. Heavier components, perhaps representing higher polymer formation, are seen as a shoulder in the lower portion of the gradient.

The formation of hybrid molecules composed of polypeptide chains derived from both γA– and γG–myeloma proteins could be demonstrated by techniques similar to those used for homologous chain recombination. H chains from γA-protein were permitted to recombine with L chains from γG-protein. Formation of the resultant hybrid molecule is shown in Fig. 2 *a*. The biphasic character of

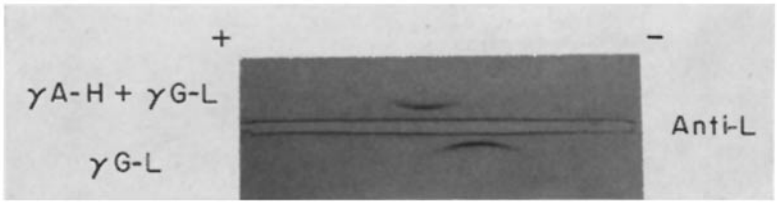


FIG. 3. Immunoelectrophoresis of the same isolated L chains in the free (lower well) and recombined (upper well) forms. Note the shift in electrophoretic mobility of the L chains following recombination. The antiserum in the central trough was prepared against kappa Bence Jones protein.

the labeled L chains is clearly shown and is similar to that seen in homologous chain recombination. Hybrid molecules were also formed using H chains from γG– and L chains from γA–myeloma proteins. These recombined molecules showed similar behavior on density gradient analysis as seen in Fig. 2 *b* and occupy the same relative position in respect to the free L chains. These experiments demonstrate the feasibility of recombination of molecules consisting of H and L polypeptide chains from two different immunoglobulin classes.

Judicious selection of both H and L chains permitted demonstration of recombination by immunoelectrophoretic analysis. L chains with a characteristically slow mobility in the free state could be shown to demonstrate a shift in mobility when recombined with H chains. Thus, as demonstrated in Fig. 3, immunoelectrophoresis using antiserum to L chain group determinants showed faster mobility of the combined chains relative to the free L chains from the same source. In these experiments an excess of H chains was used during the recombination procedure to facilitate the clear distinction in shift of L chain mobility required for this method of analysis. Antiserum to whole γA–myeloma protein showed the recombined molecules in precisely the same position as the faster migrating L chain determinants.

The possibility of preferential binding of chains derived from the same protein class over hybrid combinations was investigated in a series of competition experiments. Equal molar amounts of L chains of γG- and γA-origin were labeled with I^{125} and I^{131} respectively and allowed to combine with H chains of one class. Table I shows the ratio of γA- to γG-L chains in the resultant recombined molecules. It is clear that though there is wide variance from unity in individual experiments, no clear trend for preferential recombination of chains on the basis of their immunoglobulin class of origin emerges.

In contrast to these results is the striking degree of individual specificity for recombination of homologous chains derived from the same parent γA-mye-

TABLE I

*Nonhomologous Recombination of γA- and γG-Heavy Chains With γA- and γG-Light Chains in Competition**

γA-H	γG-H
0.7	2.3
0.5	0.9
1.2	1.3
0.3	0.5
1.7	
4.3	
0.2	
1.7	
0.9	

* Values represent ratio γA/γG light chains in hybrid molecules.

loma protein when similar L chain competition experiments were performed. Double labeling using one isotope for the homologous and a second for the heterologous L chains was again employed permitting separate analysis of the L chains in both the free and combined forms. Equal molar amounts of both L chains were added to H chains derived from one of the parent globulins. Comparison of the relative amounts of L chains in the free and recombined molecule as shown in Fig. 4 indicates the degree of individual specificity of recombination encountered in these experiments. It should be noted that both L chains are of kappa antigenic structure. Table II summarizes the results of two such experiments.

Configurational Antigenic Determinants Imposed by kappa L Chains.—In the course of studies on the capacity of kappa or lambda L chains to combine with γA-H chains, an unusual antiserum was encountered which showed specificity for kappa γA-myeloma proteins after thorough absorption with excess kappa L chains derived from a variety of sources including the protein used as the immunizing antigen. This antiserum was made in a Cynomologous monkey by

immunization with a mixture of H chains and unreduced protein derived from a single kappa γA–myeloma protein. As shown in Fig. 5 *a* sharp, distinct spurs of kappa over lambda γA–myeloma proteins are noted after absorption of the antiserum with kappa L chains. However, when isolated H chains of both

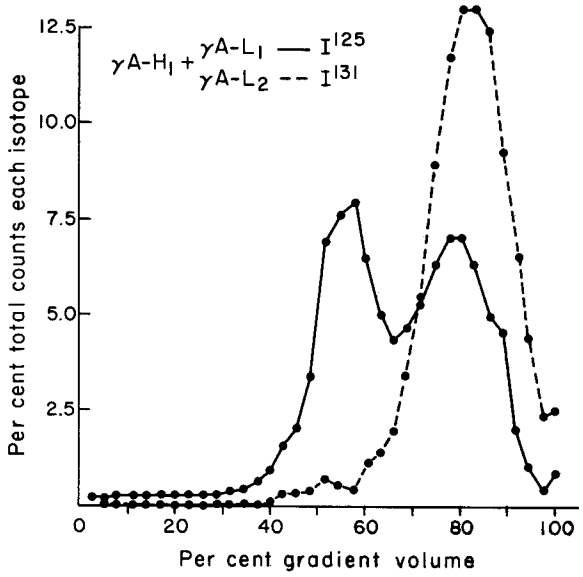


FIG. 4. Sucrose density gradient ultracentrifugation pattern illustrating preferential recombination of homologous chains in competition experiment. Note the relative per cent of homologous (L_1) and heterologous (L_2) light chains in the 7S region of the gradient.

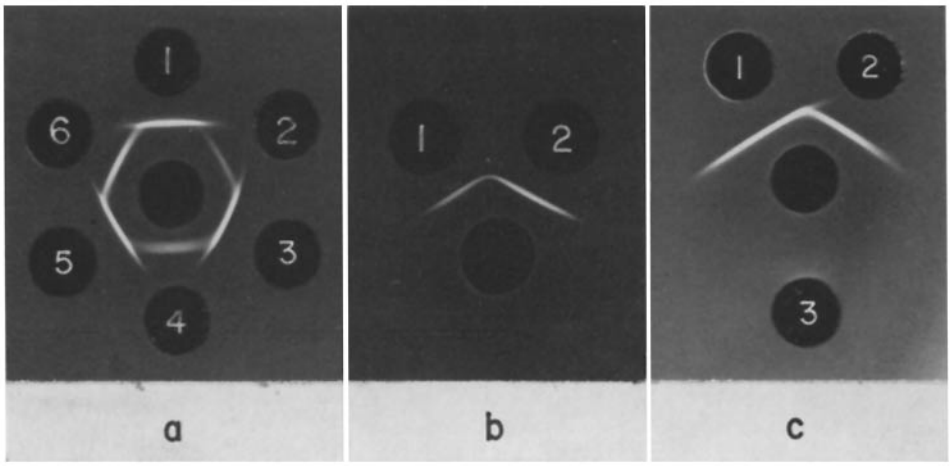
TABLE II

Specificity of Homologous Recombination of Heavy and Light Chains from γA–Myeloma Proteins

Chain combinations	Light chains in combined molecules	
	L_1	L_2
	%	%
H_1 + L_1 and L_2	49	3
H_2 + L_1 and L_2	8	27

groups were tested with the same antiserum no such spurs were observed. Immunologically and electrophoretically pure H chains derived from lambda γA–myeloma proteins were recombined with L chains from kappa myeloma proteins of either γA- or γG-origin. Such recombined molecules were immunologically identical to whole native kappa γA-molecules as shown in Fig. 5 *b*. Furthermore,

such recombined proteins spurred over the parent lambda γA-myeloma protein from which the H chain was derived as shown in Fig. 5 *c*. Experiments utilizing native kappa γG-proteins as well as recombined hybrid molecules of the type γA-L_κ–γG-H were not successful in demonstrating this antigenic determinant following absorption of the antiserum by kappa L chains. It was apparent that



FIGS. 5 *a* to 5 *c*. Configurational antigenic analysis of kappa and lambda γA-myeloma proteins and recombined H and L chains. Central wells (unlabeled) contain monkey anti-γA-antiserum absorbed with kappa L chains in each case.

FIG. 5 *a*. Comparison of three different kappa γA-myeloma proteins (wells 1, 3, and 5) with three different lambda γA-myeloma proteins (wells 2, 4, and 6) showing sharp spurs of kappa over lambda proteins in each case.

FIG. 5 *b*. Comparison of recombination of the type γA-Hλ–L_κ in well 1 and native kappa γA-protein from which the recombined kappa L chain was derived in well 2. Note the pattern of immunologic identity.

FIG. 5 *c*. Comparison of recombination of the type γA-Hλ–L_κ in well 1 and native lambda γA-protein in well 2. Well 3 contains L chains from the same source as the recombined molecule. Note the sharp spur of the recombined protein over the native lambda γA-myeloma protein and the absence of reaction with isolated kappa chains.

H chains derived from γA-proteins in combination with kappa L chains were required for the expression of this antigenic determinant. In other experiments, careful absorption of this antiserum with isolated kappa L chains and γA–H chains removed reactants to these polypeptides while preserving antibody activity against γA-Hλ–L_κ recombinations and native kappa γA-proteins. It would appear that the subdivision of kappa and lambda molecules shown by this antiserum is on the basis of configurational determinants expressed in kappa molecules within the γA-class on the basis of quaternary molecular configuration.

Character of the L chains in Double γA- and γG-"Monoclonal" Peaks from the

Same Sera.—Four sera were encountered where both a sharp γA- and a sharp γG-peak were observed in the same serum. Such sera from patients with myeloma and related disorders have been described previously (11). It was of in-

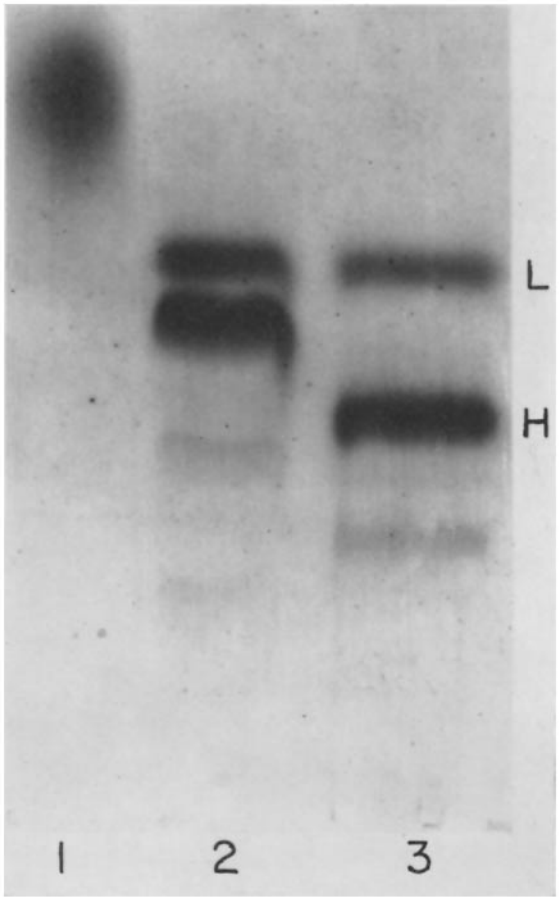


FIG. 6. Acid-urea starch gel electrophoretic comparison of isolated γA- and γG-monoclona peaks from serum AB. Origins: (*1*) L chains from Fr II γ-globulin, (*2*) γG-peak following reduction and alkylation, and (*3*) γA-peak following reduction and alkylation. H and L refer to the position of these chains for the γA-peak.

terest to study the L chains from both these components to see if they might be identical for a given serum. In the first serum studied, from a case of multiple myeloma, the L chains were found to belong to separate groups and were clearly different. The γG-peak was kappa and the γA-peak was lambda. In the remaining three sera both peaks belonged to the kappa group. These were then

studied by starch gel electrophoresis in an acid-urea gel after reduction of the isolated proteins with mercaptoethanol and treatment with iodoacetamide (9). In the case of two of the sera, strikingly different L chain bands were obtained for the γA- and γG-components. However, for the third, identical L chain bands were obtained. This result is illustrated in Fig. 6. Pattern 1 shows the distribution of the L chains isolated from Fr II γ-globulin; pattern 2 illustrates bands for the γG-peak from serum AB; pattern 3 shows the γA-peak bands from serum AB. The L chain band for both the γG- and γA-protein were discrete and had identical mobilities; both were uniquely slow and quite different from the bulk of the Fr II L chains. The H chains for the γG-protein fall just below the L chain band while the H chains for the γA-material are distinctly slower and well separated from the L chain band.

Additional experiments under varying conditions always showed the γG- and γA-components of serum AB to have L chains of identical mobility. Antigenic analyses with various kappa L chain antisera also showed that the L chains from the two peaks were indistinguishable. Both belonged to the kappa group and were also identical in subtype.

DISCUSSION

The experiments described demonstrate the feasibility of disassociating γA-myeloma globulins into their component chains under conditions closely resembling those required for the separation of the polypeptide components of γG-globulins (9). Similarly, reassociation of the L and H chains to form molecules with sedimentation and immunologic characteristics of the parent γA-myeloma proteins could be accomplished. These results provide strong inferential evidence that the noncovalent bonds responsible for the binding and positional arrangement of the component polypeptide chains in the γA-globulin molecule are similar in their physicochemical characteristics to those of the γG-class. Further evidence on this point was obtained by demonstration of recombination of individual polypeptide chains from γA- and γG-myeloma proteins resulting in the formation of hybrid molecules with characteristics similar to the native proteins from which they were derived. That these recombined molecules are similar to native 7S γG- or γA-proteins is supported by the following criteria: (*a*) the hybrid molecule occupies a similar position in sucrose density gradient to the normal intact 7S protein; (*b*) immunoelectrophoresis of the recombined chains demonstrates identity of position of the arc whether developed with an anti-H or an anti-L chain antibody whereas the unrecombined chains are demonstrated to migrate independently; and (*c*) configurational antigenic specificity, demonstrated by use of antiserum to γA-myeloma protein and dependent on the presence of kappa L chains in the recombined molecule, can be shown to exist in combinations of the type γA-H–L_κ where the L chains are of γA- or γG-origin.

Two additional observations are significant in showing similarity in behavior of γA- and γG–H chains under certain conditions. Where H chains from γA-proteins are allowed to react with equal molar amounts of L chains derived from other γA- and γG-proteins in the same reaction mixture, there was no clear-cut preference for L chains of the homologous immunoglobulin class. These results are in accord with recently published work of Grey and Mannik using γG–H chains where a similar lack of intraclass specificity was demonstrated (3), and lend support to the theory that the L chain pool is shared by all immunoglobulin classes. Another notable similarity to the results obtained in studies of γG-proteins was the marked specificity of γA–H chains for recombination with L chains derived from the same parent protein when placed in competition with other L chains of either γA- or γG-origin. Such preferential binding of homologous chains may be accounted for by the unique structure of the amino-terminal half of the L chains as recently shown for kappa polypeptides (12), or their counterparts on the corresponding Fd portion of the H chain. The interaction of these residues would then be responsible for the higher free-binding energy of the homologous pairs of chains demonstrated in competition experiments. Related results obtained with rabbit γG-antihapten antibody have been recently reported by Roholt et al. (4).

The similarity of H-L chain interactions between these two classes of proteins is not unexpected considering the well established data that L chains show similar features amongst all immunoglobulin classes (13, 14). Thus it might be predicted that production of hybrid molecules could be accomplished provided that H-L interchain–binding sites were available and not blocked by aggregation or more extensive denaturation in the process of their isolation from the parent protein. It is of special interest that a serum from a patient with multiple myeloma has been recently encountered in which both a γG- and γA-peak occur together. Isolation of these homogeneous proteins and their individual seperation in acid-urea gel following reduction demonstrated that the L chains from the two proteins showed identical mobility. Antigenic studies also indicated identity of the L chain components. It would appear, therefore, that in this instance the same L chains combined with both γG– as well as γA–H chains, perhaps in the same cells. This situation, perhaps representing "natural hybrid" formation, may be analogous to the artificial hybrids described in the present study.

The observation of configurational specificity within the γA-system and its preservation in recombined molecules is indicative of the great importance of noncovalent linkages in establishing the tertiary and quaternary structure of the molecule. It has been previously demonstrated that much of the individual antigenic specificity of myeloma proteins requires specific recombination of homologous H and L chains (15). Formation of these antigenic specificities as well as the unique type described in this study is perhaps related to the reestablish-

ment of maximal antibody specificity following recombination of H and L chains derived from antihapten antibody as shown recently in several laboratories (4, 16).

SUMMARY

The present studies demonstrate that the conditions necessary for reductive cleavage, isolation, and recombination of L and H polypeptide chains of human γA–myeloma globulins parallel those required for similar manipulation of the component chains of γG-globulin. Specificity of recombination was shown for chains derived from the same protein. In contrast, no intraclass preferential recombination was demonstrable.

Hybrid molecules, formed by reassociation of noncovalent bonds, could be synthesized from isolated chains of two immunoglobulin classes resulting in the formation of molecules of the type γA-H–γG-L and γG-H–γA-L.

Several sera containing both γA– and γG–"monoclonal" peaks were studied, one of which demonstrated the L chains associated with both peaks to be identical both by electrophoretic mobility in acid-urea gel and antigenic analysis. The possibility is considered that this case represents a naturally occurring analogue of the artificially produced hybrid molecules described in this study.

Configurational antigenic specificity of γA–myeloma proteins, imposed by the presence of kappa L chains in native and appropriately recombined molecules, provides a further indication of the importance of noncovalent bonds in the establishment of the quaternary structure of these proteins.

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