SEROLOGICAL DETECTION OF VARIABLE REGION (V_H) SUBGROUPS OF Ig HEAVY CHAINS*

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The variable or V regions of the Ig heavy chains have invariable, variable, and hypervariable sections (1, 2). The individual antigenic specificities or idiotypes (3, 4) have their localization in the hypervariable regions or near these regions (5). In the relatively invariant sections there are certain positions in the amino acid sequence, which permit the classification of the heavy chains of different myeloma proteins into three main heavy-chain variable region (V_H) subgroups. However, because of the difficulties in sequencing multiple proteins, only limited data are available on the incidence and distribution of these three different types. The aim of the present study was to establish serological typing systems for the determination of V_H subgroups in myeloma proteins and in the Ig of normal sera. Such a system would have many advantages in addition to procedural simplification; characterization of the membrane Igs of lymphocytes would be a specific example. The typing systems developed in this study utilized antisera made against myeloma proteins with known V_H subgroups as determined by amino acid sequence analysis. Certain of these antisera after absorption could be made specific for each of the three subgroups.

Materials and Methods

Myeloma Proteins. Myeloma proteins with known amino acid sequence of the V region of the heavy chain were from our own laboratories or received from other sources. Some of the myeloma proteins used were kindly provided by Doctors H. Bennich, D. Capra, M. Fougereau, and F. Putman. The proteins with known V_H subgroup based on amino acid sequence are given in Table II; many of these have been previously described (2, 6-10). A number of other proteins had only been partially typed by testing for blocked or unblocked N-terminal residues (11) or with a recently developed chemical typing system based on peptide analysis (12). In addition, a large number of myeloma proteins with known Ig class, subclass, and light-chain type but without known V_H subgroup were tested.

Myeloma proteins were initially isolated from the whole sera by starch block electrophoresis (13, 14) or by DEAE-cellulose (Whatman DE-52) column chromatography in 0.015 M phosphate buffer, pH 7.6 (14–16). In other cases the myeloma proteins were eluted from a DEAE-cellulose column by a gradient from 0.015 M to 0.3 M phosphate buffer, pH 7.6. The myeloma proteins obtained were finally gel filtrated on a Sephadex G150 column under neutral conditions to give the IgG, IgA, IgM, IgD, or IgE proteins used. The purity of the various fractions of myeloma proteins were tested by agarose gel electrophoresis after the DEAE-cellulose and G150 steps.

* Supported by Anders Jahre's Foundation for the Advancement of Science, The Norwegian Rheumatism Council, and Norske Kvinners Sanitetsforening, and by U. S. Public Health Service grant no. AI 10811 from the National Institutes of Health.

Antisera. Antisera against whole myeloma proteins, $F(ab')_2$, and Fc fragments of myeloma proteins as well as light chains from pooled γ-globulin were raised in rabbits (15). In addition, the anti-V_H subgroup antisera were raised primarily in rabbits by immunization with myeloma proteins of known V_H subgroup according to amino acid sequence analysis. One anti-V_HII antiserum (anti-Cor) was raised in a baboon. Before use, the antisera were absorbed to make them subgroup specific and to eliminate all the antibodies against the constant part of the heavy chains and the antibodies against the light chains. For example, anti-V_HII was absorbed with V_HI and V_HIII proteins (2–3 mg/ml), as well as 1–2 mg/ml of light chains. In addition, one of the myeloma proteins used in absorption was selected to have the same Ig constant region class and subclass as the myeloma proteins used for immunization. Thus all antibody activities except those directed against the V_H subgroup-specific antigens and idiotypes were presumably eliminated. For each V_H subgroup at least two different antisera were available, one made against a κ-protein and one against a λ-protein. In working with these anti-V_H-subgroup antisera in hemagglutination experiments, the myeloma proteins used for coating of red cells were always different in κ or λ from those used for immunization.

Enzymatic Fragmentation and Isolation of Heavy and Light Chains of IgG. $F(ab')_2$ fragments of IgG were prepared by pepsin digestion at an enzyme to substrate ratio of 2:100 (wt/wt) as described earlier (15). To isolate heavy and light chains, IgG was first reduced by dithiothreitol and subsequently alkylated. Then it was gel filtered under dissociating conditions in 5 M guanidine-1 M acetic acid on a Sephadex G-100 column to isolate heavy and light chains (11). The purity was controlled in hemagglutination-inhibition tests and double-diffusion tests as described before (15).

Hemagglutination Inhibition Tests. These tests were carried out with O red cells coupled by the chromic chloride method to isolated myeloma proteins of known V_H subgroup (17). The coated red cells were washed four times in sterile 0.9% saline. From a stock solution of 50 mg chromic chloride (× 6 H_2O) in 5 ml 0.9% NaCl, 0.05 ml were diluted in 0.45 ml of 0.9% NaCl. Then 0.05 ml of the last mixture was added to an equal amount of packed red cells, the tube was shaken for 1 min, and 0.1 ml of myeloma proteins (2 mg/ml) was added. The mixture was left at room temperature for 4–5 min, the coated cells washed four times with 20–40 volumes excess of saline, and used as a 1% suspension within 24 h in hemagglutination and inhibition tests as described in detail before (16–18).

Results

Specific Antisera Detecting V_H Subgroups, and Relationship between V_H Subgroups Determined Serologically and by Sequence Analysis. The anti-V_H-subgroup antisera prepared and absorbed as described above did not show precipitating activity in double-immunodiffusion experiments. They were, therefore, tested in hemagglutination and hemagglutination-inhibition tests (Tables I and II). For this purpose red cells were coated with another myeloma protein than the one used for immunization but carrying the same V_H-subgroup specificity. In this way idiotypic-specific reactions were avoided. In selecting proteins for red cell coating only those were used with a different light-chain type than the protein used for immunization, thus avoiding reactions related to heavy-chain interactions with κ-chains which are seen for the C_HI-region antigens of IgA and several IgG subclasses (19, 20). Using such procedures, specific agglutination was obtained for each of the three V_H-subgroup antisera with titers of 64–128 when tested against red cells coated by proteins carrying the corresponding V_H subgroup (Table I). In contrast, no cross-reactions between the different thoroughly absorbed V_H-subgroup antisera were observed.

The specificities of the serological V_H-subgroup reactions were further tested in hemagglutination-inhibition experiments. Thus myeloma proteins with a V_H subgroup defined by amino acid sequence analysis only inhibited in systems with the same V_H-subgroup specificity (Table II). Since both κ- and λ-proteins

TABLE I

Titers of Anti-Ig and Specifically Absorbed Anti-V_H Subgroup Antisera Against Red Cells Coated by Myeloma Proteins with Chemically Defined V_H Subgroup

Proteins used for red cell coating		Anti-Ig (R:115)	Anti-V_HI(ND)* (R:346)	Anti-V_HII(Lev)* (R:161)	Anti-V_HIII(Jon)* (R:349)
Ste	V_HI (IgG1-κ)	4,000	128	<2	<2
Ou	V_HII (IgM-κ)	2,000	<2	64	<2
Tei	V_HIII (IgG1-κ)	2,000	<2	2	64

In all systems idiotypic reactions were avoided since different proteins were used for coating and for immunization. Controls: no agglutination of unsensitized red cells or of coated red cells in PBS.

* The ND protein is V_HI, IgE-λ, Lev is V_HII, IgG3-λ, and Jon is V_HIII, IgG3-λ.

TABLE II

Inhibition of Serological V_H Subgroup Systems by Myeloma Proteins of Chemically Determined V_H Subgroup as well as Ig Chains and Fragments

		Test systems using red cells coated by myeloma protein:		
		V_HI Stu and anti-V_HI*	V_HII Ou and anti-V_HII*	V_HIII Tei and anti-V_HIII*
V_HI myeloma proteins				
ND	IgE-λ	0.002‡	–	–
Stu	IgG1-κ	0.001	–	–
Hus	IgG3-κ	0.004	–	–
V_HII myeloma proteins				
Cor	γ1-κ	–	0.002	–
Ou	IgM-κ	–	0.002	–
Lan	IgA2-κ	–	0.002	–
Lev	IgG3-λ	–	0.008	–
Herm	IgG3-λ	–	0.004	–
V_HIII myeloma proteins				
Jon	IgG3-λ	–	–	0.002
Falk	IgG1-κ	–	–	0.002
Tei	IgG1-κ	–	–	0.008
Sho	IgA1-λ	–	–	0.008
Nie	IgG1-κ	–	–	0.004
Dau§	IgM	–	–	0.002
Ig chains and fragments				
Gamma KABI		0.03	0.008	0.002
Heavy chain		0.03	0.004	0.002
Light chain		–	–	–
$F(ab')_2$		0.06	0.008	0.002

* Antisera are the same as in Table I.

‡ Lowest concentration (mg/ml) giving inhibition. –, No inhibition at starting dilution of 0.5 mg/ml.

§ This protein has certain sequence characteristics of V_HIII subgroup proteins, although initially thought to be in a new V_H subgroup (1).

belonging to a given V_H subgroup inhibited equally well, the systems were clearly not dependent on κ and λ light-chain determinants. Furthermore, no relation was seen to constant heavy-chain determinants such as Ig classes and subclasses. Also, isolated heavy chains but not light chains inhibited the anti-

V_H antisera. Pooled IgG and $F(ab')_2$ fragments showed inhibition in all the V_H systems (Table II), although somewhat weaker for the V_HI system. The results obtained by the serological vs. the sequence method showed a striking correlation. The only possible exception was with the protein Dau which by amino acid sequence analysis was thought to be either V_HIII or a new subgroup (1); it gave a strong inhibition in the V_HIII system and therefore serologically appeared to be subgroup V_HIII.

Distribution of Serologically Determined Subgroups in Proteins which had been Partially Chemically Classified by Studying Blocked or Unblocked N Terminus or by Peptide Analysis. Two other techniques have been applied for chemical determination of V_H subgroups. One is to determine whether the proteins are blocked or unblocked (11), since almost all V_HI and V_HII proteins have blocked N terminus, while V_HIII proteins usually are unblocked. Seven proteins which had been previously determined by this chemical method were studied in the serological V_H typing system. Four were V_HII and three were V_HIII. All the proteins with blocked N terminus were V_HII, and the unblocked were V_HIII.

Comparisons were also made with the results of a new and only partially defined typing system employing tryptic and chymotryptic peptides (12). Nine proteins which had been determined by this technique were studied. Eight of them showed concordant results. However, one protein which was considered as V_HIII by the chemical peptide analysis was V_HII by the serological test. This particular protein was repeatedly tested as V_HII in different serological test systems, while no inhibition was seen in different V_HIII test systems.

Distribution of Serologically Determined V_H Subgroups in Myeloma Proteins with Unkown V_H Subgroup. A panel of 131 myeloma proteins without previously known heavy-chain variable subgroup was then tested in the three V_H hemagglutination-inhibition test systems. 19 proteins (about 16%) belonged to the V_HI subgroup, 45 (about 33%) to V_HII, and 65 (about 50%) were V_HIII-subgroup proteins (Table III). In each of the groups both κ- and λ-proteins were represented. All the proteins except two were typable in one of the three V_H-subgroup systems and each reacted with only a single subgroup system. The nontypable proteins were IgG3 and IgG4 proteins, respectively. These proteins were clearly shown to inhibit IgG3 and IgG4 subclass test systems and to precipitate with anti-IgG-specific antisera in double immunodiffusion. These two proteins which were not V_HI, V_HII, or V_HIII by the test systems might represent other heavy-chain variable subgroups than the three main V_H subgroups described above, or alternatively rare genetic variants of these subgroups. Plans are underway to study these proteins in detail by chemical procedures.

Table III also lists, according to serological typing, all the available proteins studied including the 131 myeloma proteins with previously unknown V_H subgroup and in addition 36 proteins which had been classified by some chemical technique. Altogether 167 proteins are thus included and the percentage distribution of the three V_H subgroups within the different Ig classes and subclasses are listed in brackets. The percentage distribution in this total material was almost the same as in the previously unclassified group and showed V_HIII to be the predominant subgroup, representing 50% of the total while the V_HII

TABLE III

Relation between Classes, Subclasses, and V_H Subgroups in Myeloma Proteins According to Serological V_H Typing

Class	V_HI			V_HII			V_HIII			Unclassified		
	U	T	%	U	T	%	U	T	%	U	T	%
IgG1	7	12	(18)	22	25*	(37)	23	31	(45)			
IgG2	4	4	(27)	4	4	(27)	7	7	(46)			
IgG3	0	1	(5)	7	9	(47)	7	8	(42)	1	1	(5)
IgG4	1	1	(11)	1	3	(33)	3	4	(44)	1	1	(11)
Total IgG	12	18	(16)	34	41	(37)	40	50	(45)	2	2	(2)
IgA1	1	1	(5)	3	5	(28)	12	12	(67)			
IgA2				1	1	(20)	1	4	(80)			
Total IgA	1	1	(4)	4	6	(26)	13	16	(70)			
IgM	6	7	(24)	7	8	(28)	9	14	(48)			
IgD							2	2	(100)			
IgE	0	1	(50)				1	1	(50)			
Total Ig	19	27	(16)	45	55	(33)	65	83	(50)	2	2	(1)

U, myeloma proteins with unknown V_H subgroup. T, total of myeloma proteins with chemically defined V_H subgroup and myeloma proteins with unknown V_H subgroup. %, Percentage of classes, subclasses, and total Ig.

* One protein in this group was typed as a V_HIII by peptide analysis of Moulin et al. (12) but was repeatedly V_HII by serological testing (see text).

subgroup comprised 33% and the V_HI subgroup about 16% of the total Ig. Thus there was an overall ratio of approximately 1:2:3 for V_HI:V_HII:V_HIII.

The relative distribution of the V_H subgroups within the different Ig classes and subclasses is also presented. For IgG the distribution was V_HI, 16%; V_HII, 37%; and V_HIII, 45%. This is nearly the same as for total Ig, but slightly more V_HII and slightly less V_HIII. Some differences from this distribution were observed in the IgG subclasses. Particularly IgG3 had rather high percentages for V_HII and IgG2 was rather high for V_HI. Further analysis especially of the IgG subclasses is required to establish a significant relationship. In the case of IgA about 70% of the IgA myeloma proteins belonged to the V_HIII subgroup, while V_HI was low. For the IgM proteins, the distributions were V_HI, 24%; V_HII, 28%; and V_HIII, 48% indicating that V_HIII again was the major subgroup. The present work comprised too few IgD and IgE myeloma proteins to get a representative distribution pattern, but the two IgD and the IgE myeloma proteins were all found to react in one of the V_H-subgroup systems.

On the basis of the above serological V_H-subgroup determination several of the myeloma proteins not previously studied chemically, along with their specifically absorbed antisera, were tried as typing reagents. In each case these proteins could be used as coating reagents and several of the stronger antisera could be used as agglutinators furnishing hemagglutination-inhibition systems, similar to the ones described above, to detect the corresponding V_H subgroup. However, it should be emphasized that only a few of the antisera could be utilized as typing reagents. In many instances no subgroup-specific agglutination remained after the routine absorption procedure.

Relative Amounts of the V_H Subgroups in Different Human Sera. 13 normal Caucasian sera including sera from 3 homozygous Gm(a+g+) and 3 homozygous Gm(f+b+) individuals, as well as Japanese and Negro sera were investigated. Semiquantitative hemagglutination-inhibition experiments were performed with the V_H-subgroup systems that had similar sensitivity and were inhibited by positive myeloma proteins down to a concentration of 0.002–0.008 mg/ml. In all cases there was less inhibition in the V_HI system than in the other test systems. The normal sera inhibited in a maximum dilution of 256–512 in the V_HI system while inhibition was seen in dilutions from 1,024 to 4,096 in the V_HII and V_HIII systems, again with somewhat stronger inhibition in the V_HIII system. No obvious relationship was observed between the inhibitory capacity in the V_H-subgroup systems and the population groups with different Gm types.

Discussion

Previous studies have indicated that the serological determination of V-region subgroups is feasible. This was first carried out on kappa chains by Solomon and McLaughlin (21) where clear correlation between the sequence typing and serological analysis was obtained. These studies were extended and the serological typing utilized for extensive studies on kappa chains in various cross-idiotypic systems (22). Certain discrepancies were encountered in this survey that were probably due in part to ambiguities in the chemical typing analyses. Serological sub-subgroups have also been defined in the kappa chain system (22, 23). Less work has been done in the lambda system but here too evidence is available that serological methods can be used with results paralleling the typing by sequence (24).

Because of the considerable amount of sequence homology in the primary structure of proteins belonging to the same V_H subgroups, it was expected that it might be possible to establish a serological typing system for the determination of V_H subgroups. However, it proved necessary to use selected hyperimmune heteroantisera and very careful absorptions to get specific reagents. Additional precautions were taken to avoid idiotype antibodies, antibodies to genetic markers, and those to light-chain antigens as well as other determinants (20). The specificities of the V_H test systems utilized in the present study were established in several ways. Firstly, myeloma proteins chemically defined by amino acid sequence to belong to the three major subgroups became the main standard of reference. They could be classified into three groups serologically. There was complete unambiguous agreement between the sequence and serological typing except possibly in the case of one protein. Here the sequence typing was interpreted as a possible new subgroup although it had some characteristics of V_HIII; serologically it clearly fell into the V_HIII category. Secondly, a comparison was made with a preliminary chemical typing procedure using the peptide analysis method of Moulin et al. (12); here there was one difference in nine myeloma proteins analyzed. This one exception requires further work to verify the chemical typing. Also, seven myeloma proteins tested by Capra and Kehoe (11) for blocked or unblocked N terminus showed concordance with the serological system. Thirdly, in a group of 167 untyped myeloma proteins examined, with only two exceptions, all proteins could be classified into one of the three serological V_H subgroups with no overlapping. In an additional study it

has been possible to show that the same three main serological V_H subgroups are present in immune antibodies and exhibit in general a considerable restriction compared to normal γ-globulin (25).

It was perhaps somewhat surprising that virtually all proteins tested invariably were classifiable in one or another of the three V_H serological groups. This is certainly due in part to the advantage of using hemagglutination-inhibition techniques which detect only the main common antigenic features between the proteins in a given V_H subgroup. This results from the fact that only the antigenic properties shared by the myeloma proteins used for coating of the red cells and the ones used for immunization will be detected (18). In this study different coat proteins as well as different antisera gave similar results. However, exceptions may be encountered in further studies as in the V_κ subgroups (22). The two nonclassifiable proteins noted in the large scale study described above appear likely to represent a fourth subgroup. Preliminary studies with an antiserum to one of the proteins supports this conclusion.

The proteins classifiable according to the three serological V_H subgroups which included virtually all of the myelomas studied showed an overall ratio of approximately 1:2:3 for V_HI:V_HII:V_HIII. Corresponding studies on normal sera by semiquantitative titrations, indicated that the normal Ig pool includes the same subgroups in an approximately similar ratio irrespective of the population group represented. Concerning the different Ig classes and subclasses, the present study shows a distribution for V_H subgroups within IgG which was very similar to the total distribution for total Ig. Some suggestive differences from this distribution were seen within the IgG subclasses, particularly in IgG3 with somewhat high percentages for V_HII and in IgG2 with higher V_HI. For IgA there was some predominance of V_HIII but not as pronounced as that which has been described by other workers using chemical typing (11). IgM showed slightly more V_HI and less V_HII than the average for Ig. In addition to the two IgD myelomas studied another IgD protein has been partially sequenced and appears to be V_HIII (26). Thus all three IgD proteins studied so far are V_HIII. Further studies are required to establish statistical significance for any of these possible associations. At any rate the present study provides further evidence that all Ig classes and subclasses do utilize the same V_H gene pool to code for the V_H polypeptides.

The serological typing systems have several specific advantages in their applicability. They can be used to type large panels of proteins such as those described in this study to determine specific relationships to other markers. In addition, they have particular use in typing small amounts of antibodies; these can be accurately typed by the serological techniques (22) in situations where the chemical techniques cannot be used because they require larger protein samples. In the case of specific antibodies an association to antibody specificity has been demonstrated serologically in the V_κ system (22, 27). Limited sequence studies have indicated a restriction of certain antibody population to given V subgroups (28, 29). The present serological typing procedure should aid considerably in verifying and extending these associations. Finally, the serological typing system also has applicability in determining V_H subgroups in cell membranes, as for example in distinguishing the membrane Ig of B lymphocytes. Of special significance is the possibility for studying the T-cell receptor. Recent

evidence (reviewed in reference 30) from idiotypic analyses suggests that V regions of H chains are involved. If this is indeed the case, the subgroup antigens described in this study also should be present because they are determined by the framework amino acid residues intimately involved in the idiotypic antigenic sites. Such studies are currently in progress.

Summary

Serological test systems were established for determining the heavy-chain variable region (V_H) subgroups of immunoglobulin heavy chains. Myeloma proteins with known V_H subgroups based on amino acid sequence were utilized as the primary basis of reference for analysis by hemagglutination and hemagglutination inhibition. Good agreement between the chemical and serological typing was obtained and nonoverlapping systems established for the three major V_H subgroups. In a survey of 167 myeloma proteins, all except two were exclusively positive in one of the three systems. The two exceptions may represent a fourth subgroup. There was an overall incidence ratio of 1:2:3 for V_HI:V_HII:V_HIII subgroups. Some differences in the overall ratios were encountered within the immunoglobulin classes. Certain advantages of the serological typing antisera were discussed with special emphasis on their use for studies of V_H antigens on the membranes of lymphocytes.

Received for publication 16 June 1976.

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