SEROLOGICAL DETECTION OF VARIABLE REGION (Vh) 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 (Vh) 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 Vh 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 Vh 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 Vh 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 Vh 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.

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Detection of Variable Region Subgroups of Ig Heavy Chains

Antisera. Antisera against whole myeloma proteins, F(ab')2, and Fc fragments of myeloma proteins as well as light chains from pooled y-globulin were raised in rabbits (15). In addition, the anti-Vh subgroup antisera were raised primarily in rabbits by immunization with myeloma proteins of known Vh subgroup according to amino acid sequence analysis. One anti-VhII 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-VhII was absorbed with VhI and VhIII 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 Vh subgroup-specific antigens and idiotypes were presumably eliminated. For each Vh subgroup at least two different antisera were available, one made against a K-protein and one against a L-protein. In working with these anti-Vh-subgroup antisera in hemagglutination experiments, the myeloma proteins used for coating of red cells were always different in k or L 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 Vh 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 H2O) 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 Vh Subgroups, and Relationship between Vh Subgroups Determined Serologically and by Sequence Analysis. The anti-Vh 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 Vh-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 k-chains which are seen for the CmI-region antigens of IgA and several IgG subclasses (19, 20). Using such procedures, specific agglutination was obtained for each of the three Vh-subgroup antisera with titers of 64-128 when tested against red cells coated by proteins carrying the corresponding Vh subgroup (Table I). In contrast, no cross-reactions between the different thoroughly absorbed Vh-subgroup antisera were observed.

The specificities of the serological Vh-subgroup reactions were further tested in hemagglutination-inhibition experiments. Thus myeloma proteins with a Vh subgroup defined by amino acid sequence analysis only inhibited in systems with the same Vh-subgroup specificity (Table II). Since both k- and L-proteins
O. FORRE, J. B. NATVIG, AND H. G. KUNKEL 899

### Table I

| Proteins used for red cell coating | Anti-Ig (R:115) | Anti-VH I(ND)* (R:346) | Anti-VH II(Lev)* (R:161) | Anti-VH III(Jon)* (R:349) |
|-----------------------------------|-----------------|------------------------|------------------------|------------------------|
| Ste Vh I (IgG1-κ)                | 4,000           | 128                    | <2                     | <2                     |
| Ou Vh II (IgM-κ)                 | 2,000           | <2                     | 64                     | <2                     |
| Tei Vh III (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 Vh I, IgE-λ, Lev is Vh II, IgG3-λ, and Jon is Vh III, IgG3-λ.

### Table II

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

| Test systems using red cells coated by myeloma protein: | Vh I Stu and anti-Vh I* | Vh II Ou and anti-Vh II* | Vh III Tei and anti-Vh III* |
|-------------------------------------------------------|------------------------|------------------------|------------------------|
| Vh I myeloma proteins                                 |                        |                        |                        |
| ND IgE-λ                                              | 0.002†                 | —                      | —                      |
| Stu IgG1-κ                                            | 0.001                  | —                      | —                      |
| Hus IgG3-κ                                            | 0.004                  | —                      | —                      |
| Vh II myeloma proteins                                |                        |                        |                        |
| Cor γ1-κ                                              | —                      | 0.002                  | —                      |
| Ou IgM-κ                                              | —                      | 0.002                  | —                      |
| Lan IgA2-κ                                            | —                      | 0.002                  | —                      |
| Lev IgG3-λ                                            | —                      | 0.008                  | —                      |
| Her IgG3-λ                                            | —                      | 0.004                  | —                      |
| Vh III 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 Vh III subgroup proteins, although initially thought to be in a new Vh subgroup (1).

belonging to a given Vh 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-
DETECTION OF VARIABLE REGION SUBGROUPS OF Ig HEAVY CHAINS

VH antisera. Pooled IgG and F(ab')2 fragments showed inhibition in all the VH systems (Table II), although somewhat weaker for the VH I 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 VH III or a new subgroup (1); it gave a strong inhibition in the VH III system and therefore serologically appeared to be subgroup VH III.

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 VH subgroups. One is to determine whether the proteins are blocked or unblocked (11), since almost all VH I and VH II proteins have blocked N terminus, while VH III proteins usually are unblocked. Seven proteins which had been previously determined by this chemical method were studied in the serological VH typing system. Four were VH II and three were VH III. All the proteins with blocked N terminus were VH II, and the unblocked were VH III.

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 VH III by the chemical peptide analysis was VH II by the serological test. This particular protein was repeatedly tested as VH II in different serological test systems, while no inhibition was seen in different VH III test systems.

Distribution of Serologically Determined VH Subgroups in Myeloma Proteins with Unknown VH Subgroup. A panel of 131 myeloma proteins without previously known heavy-chain variable subgroup was then tested in the three VH hemagglutination-inhibition test systems. 19 proteins (about 16%) belonged to the VH I subgroup, 45 (about 33%) to VH II, and 65 (about 50%) were VH III-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 VH 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 VH I, VH II, or VH III by the test systems might represent other heavy-chain variable subgroups than the three main VH 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 VH 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 VH 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 VH III to be the predominant subgroup, representing 50% of the total while the VH II
TABLE III

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

| Class | $V_h$I | $V_h$II | $V_h$III | 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 (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 (100) |
| IgE   | 0  | 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 $V_h$III by peptide analysis of Moulin et al. (12) but was repeatedly $V_h$II by serological testing (see text).

The relative distribution of the $V_h$ subgroups within the different Ig classes and subclasses is also presented. For IgG, the distribution was $V_h$I, 16%; $V_h$II, 37%; and $V_h$III, 45%. This is nearly the same as for total Ig, but slightly more $V_h$II and slightly less $V_h$III. Some differences from this distribution were observed in the IgG subclasses. Particularly IgG3 had rather high percentages for $V_h$II and IgG2 was rather high for $V_h$I. 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 $V_h$III subgroup, while $V_h$I was low. For the IgM proteins, the distributions were $V_h$I, 24%; $V_h$II, 28%; and $V_h$III, 48% indicating that $V_h$III 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 $V_H$I system than in the other test systems. The normal sera inhibited in a maximum dilution of 256–512 in the $V_H$I system while inhibition was seen in dilutions from 1,024 to 4,096 in the $V_H$II and $V_H$III systems, again with somewhat stronger inhibition in the $V_H$III 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 $V_H$III; serologically it clearly fell into the $V_H$III 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 $\gamma$-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 $V_K$ 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 $V_H\text{I}:V_H\text{II}:V_H\text{III}$. 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 $V_H\text{II}$ and in IgG2 with higher $V_H\text{I}$. For IgA there was some predominance of $V_H\text{II}$ but not as pronounced as that which has been described by other workers using chemical typing (11). IgM showed slightly more $V_H\text{I}$ and less $V_H\text{II}$ than the average for Ig. In addition to the two IgD myelomas studied another IgD protein has been partially sequenced and appears to be $V_H\text{III}$ (26). Thus all three IgD proteins studied so far are $V_H\text{III}$. 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 $V_K$ 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
904 DETECTION OF VARIABLE REGION SUBGROUPS OF Ig HEAVY CHAINS

evidence (reviewed in reference 30) from idiootypic 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 idiootypic antigenic sites. Such studies are currently in progress.

Summary

Serological test systems were established for determining the heavy-chain variable region (VH) subgroups of immunoglobulin heavy chains. Myeloma proteins with known VH 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 VH 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 VH-I:VH-II:VH-III 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 VH antigens on the membranes of lymphocytes.

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