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Antibody Diversity Versus Antibody Complementarity

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Despite the extraordinary advances being made almost daily by recombinant DNA technology in uncovering the genetic mechanisms involved in generating antibody combining sites, we are still far from having a clear picture of how antibody specificity and complementarity are generated. Indeed, it is perhaps fortunate that the relatively imprecise term "Generation of Diversity" was introduced when the earliest amino acid sequences of the variable region of the light chain were reported and it was assumed that all diversity would be related to antibody specificity and complementarity. Not only has this proved not to be so, but newer and startling developments have identified so many different mechanisms that are generating diversity that the evaluation of the role of each in generating antibody site specificity has become extremely difficult. It is, nevertheless, of crucial importance. This review will examine critically the evidence for current views of how antibody combining sites can have so many distinct specificities.

The dissection of the antibody molecule had its beginnings nearly half a century ago when Parfentiev (117) found that diphtheria antitoxin could be digested with pepsin at acid pH to yield a product of unchanged antitoxic activity but with a molecular weight that indicated that about one-third of the molecule had been removed (120). During the same decade the development of the Svedberg ultracentrifuge and the Tiselius electrophoresis apparatus had made possible the identification of two of the five classes of immunoglobulin, IgG and IgM, and recognition by quantitative immunochemical methods (119, 69, 152) that antibody activity was associated with these proteins. Fragmentations with other enzymes such as papain were also studied, but it was not until 1958 that Porter (121) showed that papain digestion of IgG antibody produced three fragments, separable chromatographically; two had antibody combining sites and the third, which crystallized spontaneously, did not. Porter termed the two fragments Fab and the third Fc (fig. 1). We now know, of course, that the antibody used was heterogeneous or polyclonal and that with monoclonal antibody, only two fragments would be obtained, one Fab and one Fc, in a molar ratio of 2:1 thus confirming structural determinations of antibody valence on heterogeneous antibody populations (43, 90, 91) by equilibrium dialysis. With the demonstration by Nisonoff et al. (114) that digestion with pepsin gave a bivalent antibody molecule that on reduction with sulfhydryl compounds was converted to monovalent fragments (fig. 1), our correct understanding of antibody fragmentation by enzymes emerged. The next major contribution came from Edelman (37) who showed that on reduction and alkylation myeloma proteins gave two sharp bands on electrophoresis in starch gel whereas whole IgG gave two very diffuse bands. Edelman and Gally (40) identified the faster band as Bence Jones protein and termed the bands the light and heavy chains of immunoglobulins. Completely reduced and alkylated chains were insoluble and difficult to work with, but, after mild reduction and alkylation, Fleischman et al. (47) succeeded in separating heavy (H) and light (L) chains on G75 Sephadex. These developments plus the ability to obtain large quantities of Bence Jones proteins from urine, the development of the Sanger technique for determining amino acid sequence, the demonstration of the electrophoretic homogeneity of myeloma proteins and Waldenström macroglobulins (58), the insight into their monoclonality (155), the recognition of two classes of Bence Jones proteins, (99, 124), now termed κ and λ, and the discovery of the other three immunoglobulin classes—IgA, IgE, and IgD—all of which became available as monoclonal myeloma proteins, made possible amino acid sequencing. This culminated in our knowledge of the complete sequences of many immunoglobulins (39; see 88) and of the preservation in evolution of the basic IgG structure in various multimeric forms(23, 48, 49, 93, 97).

The sequencing by Hilschmann and Craig (60) of two κ Bence Jones proteins from two individuals showed the existence of the variable and constant regions and as heavy chain sequences were determined, the now generally accepted domain theory that ascribes distinct functions to each domain was put forward (38) (fig. 1). We shall consider only the variable regions of the light, V_L, and heavy, V_H, chains that form an antibody combining site and interact with the appropriate antigenic determinant. The other domains are involved with other functions of the antibody molecule, complement fixation, various receptor functions, etc. (see 49, 48, 78, 93, 97). Although it is convenient to consider the domains separately, cooperative effects between domains may occur and interactions with the antibody combining sites might transmit signals down the molecule to cell receptors and trigger proliferative events or the release of mediators that affect other cells. The carbohydrate is also uniquely distributed in a manner that separates the two C_H2

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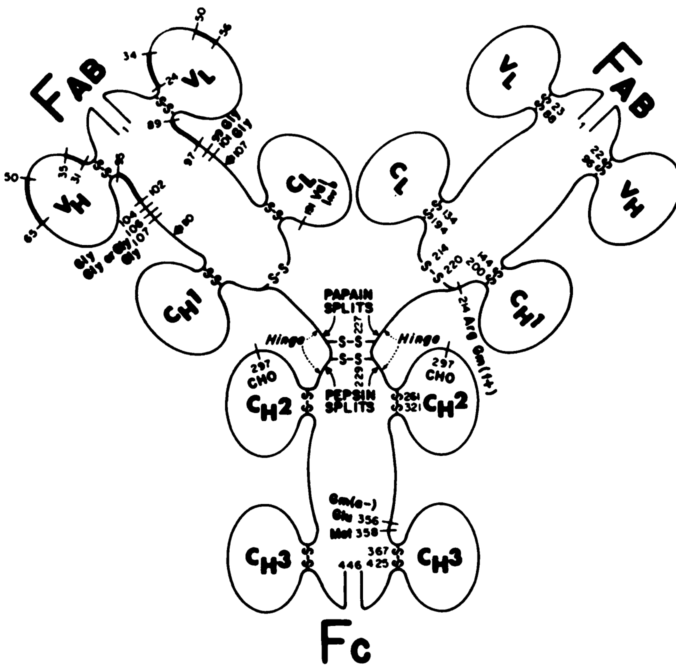

FIG. 1. Schematic view of four-chain structure of human IgG_{κ} molecule. Numbers on right side denote actual residues of protein Eu (39, 38). Numbers of Fab fragments on the left side are aligned for maximum homology; light chains are numbered according to Wu and Kabat (163, 83). Heavy chains of Eu have residue 52A and 82A, B, C and lack residues termed 100A, B, C, D, E, F, G, H, and 35A, B. Thus residue 100 (end of variable region) is 114 in actual sequence. Hypervariable regions and complementarity-determining segments or regions are shown by heavier lines. V_L and V_H denote light- and heavy-chain variable regions; C_H1, C_H2, and C_H3 are domains of constant region of heavy chain; C_L is constant region of light chain. The hinge region in which two heavy chains are linked by disulfide bonds is indicated approximately. Attachment of carbohydrate is at residue 297. Arrows at residues 107 and 110 denote transition from variable to constant regions. Sites of action of papain and pepsin and locations of a number of genetic factors are given. (From E. A. Kabat, Adv. Protein Chem., 32: 1–75, 1978.)

domains and its function should prove to be of considerable interest (148).

Another unusual feature of antibody molecules was the presence of allotypic (106) and idiotypic determinants (18, 63, 115); the former involves all immunoglobulin domains and are generally accountable as Mendelian alleles and the latter are unique antigenic determinants specificially associated with the V_L and V_H domains often related structurally to the combining site region, and generally involve both V_L and V_H for expression.

As sequencing of light and heavy chains began, it was fortunate that the human and mouse Bence Jones proteins chosen were not known to have antibody activity and thus the sequences were more likely to represent a random sample of light chains. Indeed, no human Bence Jones proteins from two individuals have been found that are identical in their V regions (125).

As the first few sequences of human and mouse Bence Jones proteins and light chains appeared (see 23), it was noted(74), after aligning them for maximum homology, that the κ and λ C-domains showed sequence variations comparable to those observed with other proteins such as the hemoglobins, cytochromes, etc., but that the variable regions were unique. Specifically, individual human and mouse V_L domains seemed more like one another than were the various human or the various mouse V_L regions. Thus there was a deficiency of amino acids that appeared to be mouse or human specific (74), e.g., species associated residues (92). There was a substantial number of invariant glycines in V_L in contrast to C_L (75) and at certain positions the number of different amino acids was much greater than at others (76, 77, 110). As more sequencing was done, the human V_L regions were divided into three subgroups (64, 110, 113). Subgroups were assigned largely on the basis of the first 23 amino acids and each subgroup was thought to be the product of a germline gene. Although the criteria for classification into subgroups permitted a few substitutions in amino acid sequence, many chains in subgroup I and in subgroup III were identical in amino acid sequence. It was pointed out (76, 77) that this segment could not be involved in generating antibody complementarity but rather constituted a framework portion of the molecule. In mouse light chains Potter (122) recognized as many as 50 subgroups based on sequences of the first 23 amino acid residues.

As more light chain sequences were published, it became crucial to attempt to locate the antibody combining site precisely. Hypotheses then current were permitting several completely unrelated antibody sites to be formed, each involving a different segment of the V region.

When 77 complete and partial sequences of human κ and λ and mouse κ Bence Jones proteins and light chains were available, Wu and Kabat (163) undertook a statistical analysis of the data. They reasoned that the antibody combining sites were all likely to be in the same position of the molecule, that the antibody forming system was universal in vertebrates, and that among sets of other nonantibody homologous proteins there was much greater variation in the framework amino acids whereas the amino acids forming the site generally showed little variation from species to species. They thus assumed that to create the enormous number of antibody combining sites in the same part of the V region from a repertoire of 20 different amino acids, one would have to have many more different amino acids at certain positions in the V region. To estimate variability at each of the positions in the V region they, therefore, devised an equation:

$$\text{Variability} = \frac{\text{Number of different amino acids occurring at any given position}}{\text{Frequency of the most common amino acid at that position}}$$

A plot of the data (fig. 2) distinguished three hypervariable regions that were hypothesized to fold to form the combining site and to contain the complementarity determining residues (163).

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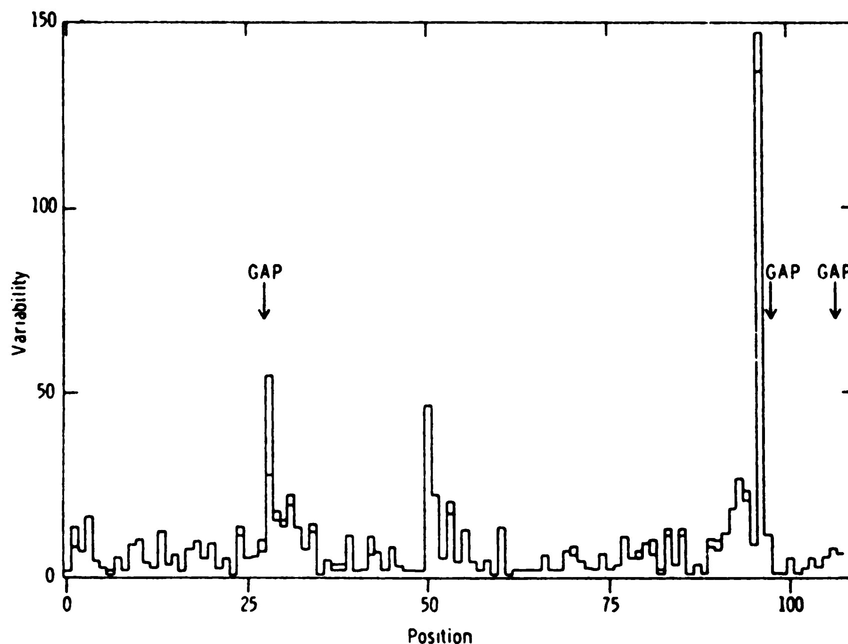

FIG. 2. Variability at different positions for the variable region of light chains. GAP Indicates position at which differences in length have been found. (From T. T. Wu and E. A. Kabat, J. Exp. Med., **132:** 211–250, 1970.)

Since the frameworks of each subgroup were then considered to be the products of a single gene, it was hypothesized that the repertoire of antibody combining sites could be generated by the insertion into the nucleotides coding for the four framework segments of nucleotides coding for the complementarity-determining segments; a similar hypothesis was favored by Capra and Kindt (17, 94).

When mouse V_λ domains were sequenced (fig. 3) (24, 158, 161), they were very restricted as 12 different V_λ chains had an identical sequence throughout the V region; this was considered to be the germ-line sequence. Six others had from one to three substitutions all, with one exception at residue 48, confined to the hypervariable regions as originally defined (163). Since these, including the substitution at position 48, could be derived from the germ-line sequence by single-base changes, this was taken as evidence that somatic mutation (see 111) in the hypervariable regions was generating the diversity and by inference complementarity differences. It was subsequently pointed out that all mouse V_λ domains had an insertion of three residues in the first hypervariable region (80) and, thus, that in V_λ chains the second hypervariable region would consist of residues 48 to 56 and in the other chains 50 to 56.

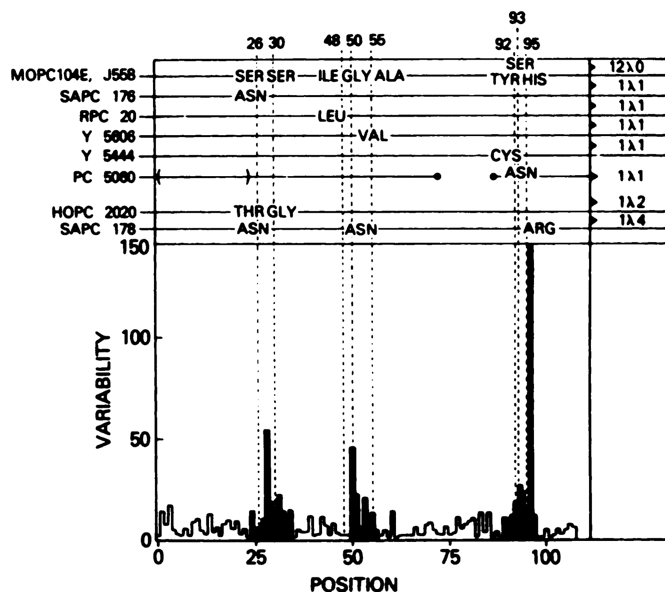

FIG. 3. Location and nature of amino acid substitutions in the mouse V_λ region superimposed on the variability plot of figure 2. The three complementarity-determining regions according to Wu and Kabat (163) are shaded. All other residues in the sequences were identical. The number of instances of each sequence as well as the number of base changes to obtain it from λ0 are given [From E. A. Kabat, Adv. Protein Chem. **32:** 1–75, 1978; data from Cohn et al. (24), Weigert and Riblet (161)].

As V_H sequences become available the variability plot of heavy chains of all species available identified three corresponding hypervariable regions (83) comparable to the three in the light chain (fig. 4). Somewhat increased variability was originally seen at positions 81 and 83 (83). Capra and Kehoe (15, 16), restricting the variability plot to human V_H regions, termed this an additional hypervariable region involving positions 84 to 91. However, as more data accumulated, the hypervariability of this segment, whether on all V_H chains or on human V_H chains, disappeared (fig. 4) (88).

Affinity labeling studies from several laboratories (see 52) showed that only residues in the hypervariable regions were labeled thereby confirming that they lined the

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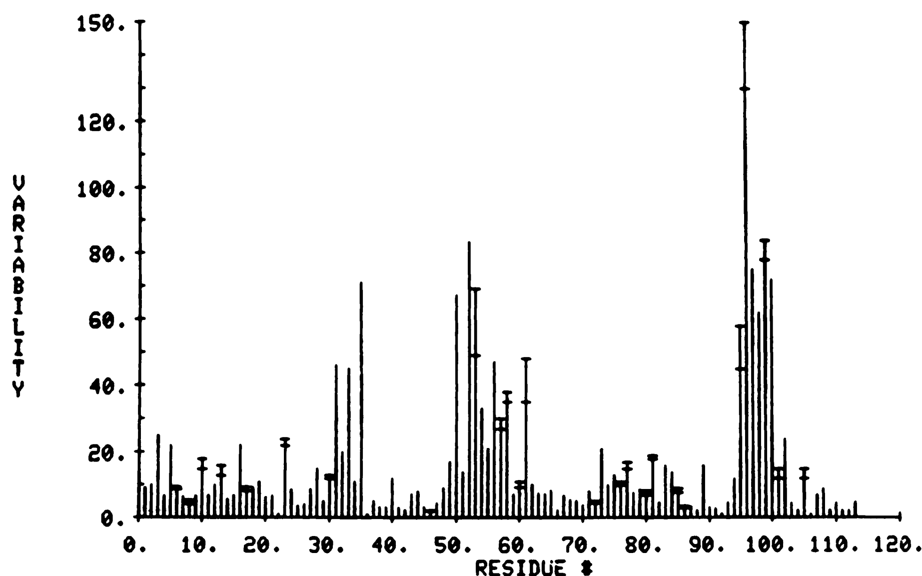

FIG. 4. Variability at different positions for the variable region of heavy chains. The plot was made by the PROPHET computer system (127). (From E. A. Kabat, T. T. Wu, and H. Bilofsky, Government Printing Office Publication NIH 80-2008, Washington, D.C. 1979.)

walls of the antibody combining site; when x-ray crystallographic studies of Fab fragments, light chain and V_L dimers (3, 31–33, 41, 44–46, 137, 142, 157) became available, they confirmed on a residue for residue basis the prediction (163) that the three hypervariable regions of V_L and V_H would form the walls of the antibody combining site. Residues 84–91 (15, 16) were at the back of the molecule remote from the site. Figure 5 shows stereopairs of the carbon skeletons of four of the six X-ray crystallographic structures with the hypervariable regions circled (80). As a consequence of the finding that the three hypervariable regions in each chain actually form the walls of the site and to avoid confusion with residues 84 to 91, the hypervariable regions have been termed complementarity-determining regions (CDR) (80), with the rest of the V regions constituting a framework (FR). This was not intended to exclude the possibility that FR residues could influence the site since, it was stated in the initial description of hypervariable regions (163) "that binding could be influenced to some extent by different residues adjacent to a site but not in themselves complementarity determining." Moreover, it was never intended that every residue in each CDR should either be contacting or conformationally influence a contacting amino acid. Indeed, the nature of the peptide bond and of side chain conformations precludes interaction on the inside of an antibody combining site of each of the side chains in a CDR of 7 to 12 or more sequential residues. An extensive subsequent analysis of the various amino acids at each position in the CDR (85) together with the x-ray crystallographic data has defined certain amino acids at certain positions as playing a structural rather than a contacting or complementarity determining role and these data were in accord with the four x-ray crystallographic structures available at the time; 13 positions in V_L and seven in V_H were so defined; the V_H sequence data was more limited. As more and more sequences began to be accumulated on groups of monoclonal myeloma proteins followed by an increasing number of individual hybridoma proteins of one or another specificity, the randomness of the collection may have become somewhat distorted (79) and will continue to become even more distorted. This has unfortunately led Cohn et al. (25) to state that, "this type of analysis is inherently unable to provide a criterion of whether a given residue in a given V region is FW [read FR] or CD [read CDR]." Obviously, dilution of the original more random data by selected groups of antibodies from myeloma and hybridoma antibodies that would naturally have similar CDRs would eventually eliminate much of the variability. The data and the residues defined by the original hypervariability plots (163, 83, 85, 88) as CDR should continue to be used to study site structure.

A major difficulty in attempting to map sites from existing x-ray data derives from the insertions and deletions in or adjacent to the CDR. In V_L of McPC603 an insertion of six residues in CDR1 shields CDR2 from the site (142) and in Newm a deletion of seven residues in V_L after CDR2 removes it completely from the site region (3, 137). For a functional chain to exist, CDR2 would become part of the framework. Thus, it should not have been surprising to Cohn et al. (25) that, "In fact no positions in CDR2 are defined as contact residues by x-ray analysis." In chains with no deletions or insertions, CDR2 clearly contains contacting residues as seen from analysis of the x-ray data on light chain dimers as well as an Fv dimer and in a model of type III antipneumococcal antibody constructed (33) by replacing the CDR of

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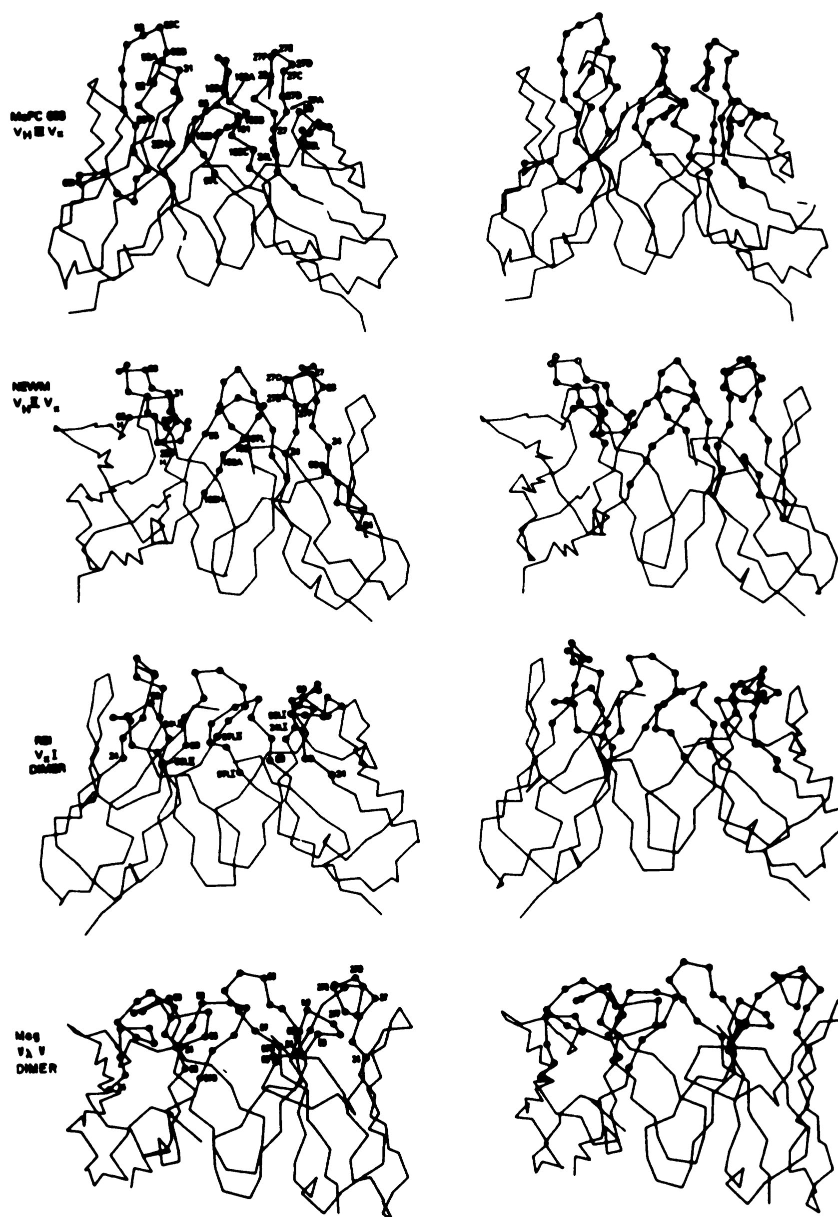

FIG. 5. Stereodrawings of the α-carbon skeletons of the V regions of four of the five proteins studied crystallographically. Each protein is in the same orientation. With a stereoviewer it is possible to see two adjacent models at the same time, so that a comparison may be made in three dimensions. (From E. A. Kabat, Adv. Protein Chem., 31: 1–75, 1978.)

McPC603 with the CDR of type III antipneumococcal antibody. One must also bear in mind that differences in length of the CDR may play a major role in antibody complementarity since they may permit the same amino acid in a homologized position as seen in the variability plot to function slightly displaced in the site and thus generate a different specificity.

Unless substantial efforts are made to crystallize and study myeloma and hybridoma antibodies of defined specificity and with ligands that completely fill the site, progress in understanding antibody specificity and complementarity will be extremely limited. Unfortunately, in neither of the two Fab fragments (137, 142) studied crystallographically is a ligand known that fills the site completely and the V_L dimers interact with a variety of structurally very different ligands (41).

In the absence of several high resolution x-ray crystallographic structures in which ligands completely filling the site are available, one has had to depend on the mapping of antibody combining sites by quantitative immunochemical methods and attempts are being made to correlate these findings with sequences, idiotypic specificities, and other parameters (80, 85) in the hope that ultimately those inferences will be validated or modified when certain of these myeloma and hybridoma antibodies are crystallized. Monoclonal myeloma antibodies to homopolysaccharides (19, 20, 6, 9, see 123) have provided the most insight into mapping antibody combining

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sites and this is being substantially augmented by hybridoma antibodies (146, 147). The first and most extensively studied system was that of α1→6 linked dextran (70–73, 19, 20, 6, 9) and its homologous antibodies, followed by studies on α1→3 linked dextrans (105), β1→6 linked galactans (9, 53), and β2→6 fructosans (105, 53).

Almost three decades of work on the α1→6 dextran-antidextran system resulted in: 1) the demonstration that dextran was antigenic in humans (82, 107); 2) the availability of a dextran with 96% of α1→6 linkages and 4% of α1→3 linkages as branching points (66) most of which contained but one or two glucose units with only a small proportion having long branches (30, 102); and 3) the isolation of the isomaltose series of α1→6 linked oligosaccharides from the disaccharide to the heptasaccharide (67, 154), and more recently of the octasaccharide (see 146), provided a molecular ruler for probing the size of the antibody combining site. The approach involved quantitative inhibition by the various oligosaccharides of the precipitin reaction between dextran and the human α1→6 antidextran, on the assumption that, on a molar basis, the best inhibitor would be the isomaltosyl oligosaccharide filling the site completely and oligosaccharides of greater chain length would not show increased inhibiting power. These studies with heterogeneous human anti-α1→6 dextrans established the upper limit for an antibody combining site to be complementary to six and seven α1→6-linked glucose residues (70–72; see 73, 78), the former measuring in its most extended form 34 × 12 × 7 Å, and the latter, about 4 to 5 Å longer. The lower limit was between one and two α1→6-linked glucose residues (4, 150). Linear oligosaccharides containing non-α1→6 linkages in the main chain or branches on linear chains of α1→6-linked residues reduced accessibility to the antibody combining sites as measured by lower inhibitory potency and compared to the linear α1→6 linked oligomers. Thus, over the years, not only were the anti-α1→6 dextran sites completely mapped but antidextrans from single individuals could be fractionated into subpopulations with larger- and with smaller-sized combining sites (141, 51, 54, 166). Studies on a wide variety of synthetic and natural antigens of all types gave combining site sizes within the range found for α1→6 antidextran (see 78).

An important additional insight came with the study of monoclonal mouse myeloma antibodies with specificity for α1→6 dextrans (19, 20). These offered the striking advantage that one was no longer dealing with heterogeneous populations of antibody combining sites, but with the same techniques one could determine the sizes of the combining sites of homogeneous antibodies and by comparing one with another be able to learn something about the kinds of antibody sites specific for α1→6 dextran. Two antidextran myelomas provided an unusual insight. One, W3129, was specific for the nonreducing ends of the dextran chain and had a site size complementary to isomaltopentaose with methyl α-D-glucoside and isomaltose contributing a substantial proportion of the total binding energy relative to that of the pentasaccharide as measured by inhibition with oligosaccharides, by equilibrium dialysis, and by fluorescence quenching. W3129 has an association constant K^a for isomaltopentaose of 1×10^5 liters per mole.

The second, QUPC52, was found to be specific for internal chains of α1→6-linked glucoses with site size complementary to IM6, e.g., six α1→6-linked glucoses. Its K^a was only 8×10^3 liters per mole and methyl α-D-glucoside and isomaltose contributed less than 5% of the binding energy of the IM6. W3129 was interpreted as having a cavity-type site with the terminal one or two glucoses at the nonreducing end being held in the site in three dimensions whereas QUPC52 was considered to have a groove-type site open at both ends (20). A very rapid and definitive method for distinguishing these two kinds of sites was available since Ruckel and Schuerch (132) had synthesized a completely linear dextran with about 200 α1→6-linked glucose units. This linear dextran, D3, and a similar product LD7 prepared by Ito and Schuerch (see 146), did not precipitate with the cavity type site of W3129 but, since D3 was monovalent with one nonreducing end, inhibited its precipitation by dextran about as well as IM5. However, D3 was multivalent with respect to QUPC52 with groove-type sites and actually precipitated with it (20). Thus, with monoclonal antibodies the distinction between cavity-type and groove-type sites could be made very simply based on the presence or absence of precipitation with the linear dextran. Bennett and Glaudemans and others (6, 9, 131) confirmed the cavity-type nature of the W3129 site by using Fab fragments, and it has also been verified by affinity electrophoresis. The concept of cavity-type and groove-type sites has been extended to antibodies to A-variant streptococci (138), to anti β1→6 galactans, (9, 131), and to the anti-α1→3 dextran MOPC104E (139).

By applying the hybridoma technique to the α1→6 antidextran, Sharon et al. (145) developed the replica immunoadsorption technique (144) by which they isolated 12 α1→6 antidextran clones by screening 100,000 hybrids (145–147). All of the 12 differed in one or more of the various parameters including size of combining site, association constant by affinity electrophoresis, idiotypic specificity, relative contribution of each sugar unit to binding, and translucence or opacity of the dextran antidextran precipitates. Seven were IgMκ and five IgAκ. Cloning and sequencing DNA from these hybridomas and attempts to crystallize them and study their sites, if successful, should contribute materially to the understanding of antibody complementarity and specificity and to the mechanism of its generation. Another recent development in the dextran α1→6 antidextran system is the synthesis by Charles Wood in my laboratory of a set of synthetic isomaltosyl glycolipids by coupling the isomaltose oligosaccharides from IM2 to IM7 to stearylamine (162) by using cyanoborohydride (56). These glyco-

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lipid antigens of completely defined size and structure are antigenic in rabbits whether administered with Freund complete adjuvants alone or on liposomes. The predominant response involved formation of cavity-type rather than groove-type sites (162a, 162b).

These products not only make possible study of the genetics of the antibody response to glycolipids in various animals, especially in inbred lines of mice, but, also, by replica immunoabsorption (144) one should be able to obtain hybridomas with cavity-type sites of various sizes for comparison with the groove-type hybridoma sites, for studies by recombinant DNA technology, and by high resolution x-ray crystallography.

We should thus have a series of immunochemically characterized combining sites of defined sizes, shapes, and specificities together with sequences and, hopefully, several crystallographically characterized sites; this should permit a detailed understanding of the variations in site size and structure in relation to sequence. This, together with the sequencing of the corresponding genomic and plasmacytoma DNA clones responsible for the various antibodies, should permit analysis of the extent to which the various genetic mechanisms to be discussed below contribute not only to the generation of diversity but also to the generation of antibody complementarity. This will also provide insights into the efficiency of the process and the extent to which generation of diversity may be wasteful and noncontributory to the making of antibody complementarity.

A cooperative attempt in this direction is being made with $\alpha 1\rightarrow 3$ antidextran hybridomas by Barbara Newman and Shunji Sugii in my laboratory, by Mitsuo Torii in Osaka, Japan, and by Brian Clevinger and Joseph Davie at Washington University; the V_H regions of these hybridomas were sequenced by Jim Schilling and Leroy Hood (140). The heavy chains were remarkably uniform in sequence through residue 95 except for substitutions of Lys for Asn in one instance, but varied beyond this point. Variation was most extensive at positions 96, 97, and 98* and less marked beyond this point with many residues being invariant. These data together with nucleotide sequencing of genomic V_H clones led to the recognition of D and J minigenes in V_H (see below) (36). Since the light chains are all λ, their V regions would be expected to be relatively similar although the absence of sequence data makes results tentative.

With respect to their antibody combining sites, they fall into several groups in their reactivity with various dextrans as seen in quantitative precipitin curves. Three class II dextrans, known to contain the highest proportion of $\alpha 1\rightarrow 3$ linkages (143, 112) and to be highly branched with the predominant chain structure being alternating sequences of $\alpha 1\rightarrow 6$- and $\alpha 1\rightarrow 3$-linked glucoses, reacted most strongly with all of the hybridomas. However, by using 20 or more other dextrans of class I built of long chains of $\alpha 1\rightarrow 6$-linked glucoses with monoglucosyl branches linked $\alpha 1\rightarrow 3$, $\alpha 1\rightarrow 4$, or $\alpha 1\rightarrow 2$, precipitin curves fell into several patterns. In several instances cross reactions occurred with considerably more of certain dextrans being required to precipitate equivalent amounts of antibody. The relative extent of this cross reactivity varied from hybridoma to hybridoma. However, two hybridomas reacted only with the three class II dextrans and not at all with any of the other 20 dextrans (66) used. When these two hybridomas were compared in sequence they differed in CDR3 (D and J segments) in all but one amino acid residue (140). In the absence of light chain data one may hypothesize that the portion of the site involved in the cross reactivity with class I dextrans was blocked by the side chains of amino acids in CDR3 of V_H; also, in view of the differences in amino acid sequence, that inaccessibility to part of a site may be nonspecific in that different amino acid side chains may create an equivalent steric block. This inference sheds a new light on antibody complementarity in that similar effects on a site may result from different amino acids with diversity in sequence not necesarily being an indication of functional differences in site complementarity. Although these findings must be considered as tentative until supplemented by inhibition assays with oligosaccharides, measurements of binding constants, and sequencing of the light chains, they nevertheless reveal a hitherto unsuspected aspect of antibody complementarity.

The introduction of recombinant DNA technology has effected a revolution in our understanding of the genetic aspects of protein structure. The discovery of intervening sequences has completely changed our understanding of evolution at the molecular level. Nowhere has this been more evident than in studies of immunoglobulins (1). Intervening sequences are found between the domains of immunoglobulin chains. They separate the genes coding for the various domains of immunoglobulins (35). They have recognition sequences involved as splice sites for the joining of C-region domains in heavy and light chains and as switch sites for the change in synthesis from IgM to the other classes (29). Much of this, to the extent that it involves the splicing out of intervening sequences to assemble the C-regions, is also seen in the genetic control of other proteins such as hemoglobin (68, 103, 104, 151), conalbumin (21), etc. Unique genetic mechanisms are involved in the shift in synthesis from membrane IgM to secreted IgM (156) and in the assembly of L and H chains (34, 62) and of the V regions (36, see 129). It is this latter aspect that is crucial to our understanding of the relation between antibody diversity and antibody complementarity.

The initial clue that the genome of the variable region was unique was implicit in the findings by Hozumi and Tonegawa (65) that in embryonic DNA the V and C regions were separated by a greater distance than in plasmacytoma DNA. Cloning and nucleotide sequencing

* Numbering of amino acid residues according to reference 88.

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of a V_λ gene obtained from 12-day-old mouse embryo DNA by hybridization with a V_λ I mRNA showed that there was a precursor sequence followed by an intervening sequence of 93 nucleotides and a sequence coding for the variable region from amino acid −4 through amino acids 95 or 96. Beyond this, the nucleotide sequence did not code for amino acids known to comprise the rest of the V region corresponding to Fr4 (153).

At this time Drs. Wu, Bilofsky, and I had been compiling the amino acid sequences of V regions of light and heavy chains in the PROPHET computer system supported (127) by National Institutes of Health and arranging the data into sets of identical FR segments. At that time only two pairs of human V_L regions each with an identical CDR had been described (164, 95) and these data tended to support an insertional mechanism. More recently, a pair of unrelated rabbits with identical V_L regions has been found (13).

When the available human V_κI, mouse V_κ, and rabbit V_κ sequences were grouped separately, each into sets of identical FR1, FR2, FR3, and FR4, evidence of independent assortment of these FR segments emerged (86). Thus in figure 6, there were three identical sets of FR1 in V_L chains, one with 18, another with 7, and a third with 2 members plus numerous sets with only one sequence; each set differed in amino acid sequence in at least one residue. Sets of FR2 and FR4 with more than a single member were also found. When each chain was traced, it was observed that members of the same set in FR1 could belong to different sets in FR2, etc. Similar patterns were observed for mouse V_κ, rabbit V_κ, and for human and mouse V_H chains (86). Moreover, in human V_κ chains it was found that FR4 appeared independent of subgroups confirming the observation by Milstein in 1967 (110) that his three human subgroups could be traced only through position 94 as if recombination was occurring beyond that point. Thus, several sets of human FR4 had members of more than a single subgroup; one had two members of V_κI, two of V_κII, one of V_κIII, and one of V_κIV (86).

Knowing that the V_λ clone from 12-day-old embryo coded only through amino acids 95 or 96 (153), and that the Bence Jones protein from the adult plasmacytoma coded for the entire V region, it was hypothesized (86) that the intact V_λ chain was assembled somatically by adding the nucleotides coding for FR4 to those coding for the rest of the V region by recombination sometime between day 12 of embryonic life and the adult myeloma.

It was further hypothesized from the assortment data that the FR segments could be considered to be minigenes and that the diversity in the V region could be accounted for by assortment of germ-line minigenes coding for the FR and by implication of the CDR segments. Since the CDR segments had not been included in the analysis it could not be excluded that one or two residues of a CDR assorted with a FR segment (87).

The V_λ clone (Ig13λ) of Tonegawa et al. (153) was

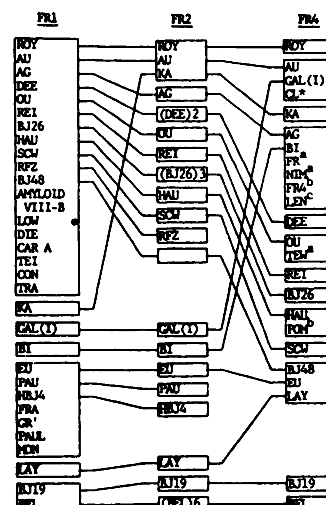

FIG. 6. Independent assortment of framework sets in human V_κI chains. Solid circle, cold agglutinin with antiblood group I activity; a, human κ light chain subgroup II; b, human κ light chain subgroup III; c, human κ light chain subgroup IV. (From E. A. Kabat, T. T. Wu, and H. Bilofsky, Proc. Natl. Acad. Sci. U.S.A. 75: 2429–2433, 1978.)

considered to be V_λII although V_λI had been used in the hybridization. The nucleotide sequence of the cloned V_λ was determined. In FR1, the amino acid sequence of V_λI and V_λII differed at four positions all of which coded for V_λII; in FR2, V_λI and V_λII differed at one position and the nucleotide sequence coded for V_λI; and in FR3, V_λI and V_λII differed at four positions and the nucleotide sequence coded for the amino acids in V_λII at all four. Although the finding in FR2 was ascribed to somatic mutation (153), it was pointed out (86, 81) that it could also have been a double recombinant with the FR1 and FR3 segments having V_λII and the FR2 segment being V_λI, consistent with the assortment data. There were also nucleotide differences in the CDRs some of which coded for V_λI, others for V_λII, and one for an amino acid seen in neither.

Shortly thereafter three additional DNA clones were isolated and sequenced (8, 11): Ig99λ and Ig25λ from 12-day mouse embryo and Ig303λ from adult V_λI plasmacytoma H2020. By DNA sequencing, it was seen that Ig99λ coded for a precursor and an intervening sequence followed by the V region sequence from residues −4 through residue 95; Ig25λ coded for residues 96 through 107, the last two residues of CDR3 plus FR4 termed the J segment, followed by an intervening sequence of 1250 bases and the nucleotides coding for the C region. The DNA from the adult plasmacytoma Ig303λ coded for the precursor and the intervening sequence, the entire V region, an intervening sequence identical to that of Ig25λ followed by the nucleotides coding for the C region. The assortment of FR4 and the hypothesized somatic assembly (86) by recombination at the DNA level of the J segment (two residues of CDR3 + FR4) were thus confirmed.

In mouse V_κ21 chains, Weigert et al. (159) used the

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assortment principle to show that a similar J segment of two residues of CDR3 + FR4 assorted independently of the rest of the V region and suggested that position 96 in J could contribute to the generation of diversity by assortment with different V regions. Rao et al. (126) proposed a similar J segment for the V_H region of the antigalactan binding proteins.

By R-loop mapping Brack and Tonegawa (12) had presented data in 1978 to show that on a genomic clone there were five J_κ segments each separated by short intervening sequences followed by a long intervening sequence and the nucleotides coding for the C region. Cloning and nucleotide sequencing by Max et al. (108) and by Sakano et al. (134) defined the five J_κ segments, J1 through J5 with J1 at the 5′ end. Four of the J segments coded exactly for amino acid sequences of residues 96 to 108 in myeloma proteins; the fifth, J3 is considered not to be an expressed gene; it differed in nucleotide sequence from the other four J minigenes in having CT rather than the GT usually found at the border of all coding sequences and associated with splicing out of intervening sequences. Moreover, it differed from all known κ and λ chains in having Ser at position 99, instead of the usual invariant Gly present in the 149 sequenced chains, and an Asp at position 100 and Pro at position 108, which also have never been reported (88). It is thus questionable as to whether it could form a functional chain even if splicing could take place. Since position 96 was in the CDR and since there were eight distinct J segments in mouse V_κ chain and only four functional J genes, it was proposed (108, 134, 160) that additional sequence diversity could be generated by recombinations within the nucleotides of the recombining codons, e.g., between the noncoding nucleotides in the V region corresponding to position 96 and the nucleotides coding for position 96 in J. This hypothesized intracodon recombination accounted for some of the additional amino acids reported for position 96 especially since one V_κ21 protein had an extra Pro residue at the V-J junction (159). However, Rudikoff et al. (133) have found five anti-β1→6 galactan specific mouse myeloma proteins to have an Ile at position 96 and this could not be generated by any of the hypothesized intracodon recombinations. A sixth had Trp at this position.

The presence of five Ile and one Trp at position 96 in these β1→6 galactan specific myeloma proteins raises the important question of whether sequence diversity at position 96 necessarily creates differences in complementarity and site specificity. Thus, the antigalactan with Trp 96 and one of the antigalactans with Ile 96 showed about the same association constants and behaved similarly in binding 30 different haptens; the five antigalactans with Ile 96 all differed in association constants. Clearly in these five proteins the differences at position 96 are not involved in the generation of antibody specificity (133). The only myeloma protein in which position 96 is involved as a contacting residue is in McPC603 in which it is involved in binding phosphorylcholine (142). The extent to which the amino acid sequence diversity generated by the proposed intracodon recombination contributes to antibody complementarity and the role of position 96 requires further study.

An important difficulty in evaluating binding in antibody combining sites derives from the findings of Firca et al. (46) that the Bence Jones Mcg dimer and a covalently linked light chain dimer derived from the intact myeloma protein Mcg were indistinguishable chromatographically, electrophoretically, and in binding dinitrophenyllysine, whereas the intact Mcg immunoglobulin does not bind dinitrophenyllysine, nor did the H_2L_2 molecule assembled from the myeloma protein heavy chain and the urinary Bence Jones light chain. Thus, the specificities of the light chains themselves are not indicative of their true specificity when combined with their heavy chains.

A further difficulty, which must be clarified for the generation of complementarity, derives from the studies of Klein et al. (96) who found that only autologous V_κ fragments recombined with the Fd′ fragment (V_H–C_γ1) of the heavy chain, but that if the C_κ fragment, although separated from V_κ, was bound to the Fd′, heterologous V_κ domains would then bind. Thus, the true degree of selectivity of autologous V_κ and V_H chains may be greater than has been anticipated from autologous and heterologous L-H chain recombinations. This would tend to argue against the often made assumption that the repertoire of antibody combining sites is generated by random L and H chain recombination (e.g., 10^3 H × 10^3 L ≅ 10^6 antibody sites).

Peabody, Ely, and Edmundson (118) have been able to make hybrid V_L dimers in good yield one of whose parent dimers, Mcg, had been studied crystallographically (41) to obtain crystals, to demonstrate that the hybrids had different binding properties, and to relate this to contacting residues in the site. It will be important to attempt to make and crystallize V_L-V_H hybrids and to study changes in site structure. This might provide data on the validity of random V_L-V_H association in the generation of complementarity.

It was well known that even intact heavy chains showed substantial preference in reassociating with their autologous light chains when presented with a mixture of heterologous and autologous chains (57). This was not due to hysteresis since with anti-α1→6 dextran specific hybridomas, which produced the specific as well as the parent κ light chains, there was invariably preferential assembly of the autologous light chain with the heavy chain (145).

Turning now to the cloning studies on V_H genes, Early et al. (36) identified and sequenced germ-line J_H genes, J_H1 and J_H2, and Sakano et al. (136) and Gough and Bernard (55) sequenced four J_H genes; J_H3 and J_H4, the two additional ones, were 3′ to J_H2. Each J_H gene was separated by small intervening sequences of several

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hundred nucleotides and J_H4 was followed by an intervening sequence of about 8 Kb, 5′ to the coding segment of the $C\mu$ chain. The J_H minigene also contained several residues of CDR3 plus FR4. When cloned V region genes were sequenced, the coding regions ended at the end of FR3. When the sequences of the V region and the J_H germ-line genes were examined, nucleotides of five amino acid residues of CDR3 were missing (36). They could not be found either on the 5′ side of J or the 3′ side of V. Indeed, the coding regions were preceded and followed by palindromic heptanucleotide CACAGTG and decanucleotide GACACAAACC sequences with spacers of 11 or 12 and 22 or 23 nucleotides that were also found adjacent to coding regions of the κ and λ light chains (36) and that are considered to be involved in joining J_L to V_L. Subsequently Sakano et al. (136) found another missing segment of 14 amino acids. These missing segments representing the major portion of CDR3, which can vary extensively in length, would appear from the x-ray structures (fig. 5) to make a substantial contribution to antibody complementarity and were termed "D" for diversity segments. Since D segments were contiguous neither with J or with V, they too were minigenes as inferred from the assortment data (86, 87, 89, 81). Most recent studies by Sakano et al. (135) and Kurosawa et al. (100) have located three distinct D segments, one coding for three and the others for four amino acid residues. Most significant is the finding that on each side of the D coding segment are similar palindromic sequences with a 12 base pair spacer; since the V_H coding region and the J_H coding regions both have 23 base pair spacers, the rule formulated by Early et al. (36), that joining of V and J in κ and λ chains involves a 23 and a 12 base pair spacer, is preserved in that the V-D-J junction involves the V23-12D12-23J spacers. It is of significance that 11 or 12 and 22 or 23 base pairs constitute one and two turns of the DNA helix respectively, thus, perhaps providing the orientation for recombination. Tonegawa has recently proposed that length differences in CDR3 may be a consequence of joining of several D minigenes to produce longer CDR3 segments (S. Tonegawa, ICN-UCLA Symposium, Feb. 8–15, 1981, Salt Lake City, Utah; see 129).

There is thus no longer any question that germ-line J_L, J_H, and D_H minigenes are involved in the assembly of complete V regions and that they are assembled somatically during development as postulated from the assortment data (86, 87, 89). Further studies are needed to establish the extent to which this assembly process generates antibody complementarity and specificity. This will become clear only as genetic, immunochemical site mapping, sequencing, and x-ray crystallographic data become available on antibody combining sites of defined specificity.

If we now turn to the rest of the V_L and V_H genomes, there are two major lines of data that must be reconciled. It is clear that in clones from sperm, 12- or 13-day embryo, liver, etc., numerous investigators have established that the nucleotides coding for V_L excluding J (8, 7, 101, 153) and for V_H excluding D and J occur as contiguous stretches (10, 28, 36, 62, 136, 167). On the other hand there is substantial evidence of assortment of FR and CDR segments (86–89). Indeed, in the rabbit it has been possible to assort all FR and CDR segments (89). Moreover, the nucleotide sequences of κ and λ chains had short stretches of identical nucleotide sequence bounding the FR and CDR segments that could serve as sites for assortment by recombination of FR and CDR segments (165). Most striking was the finding that a match of six nucleotides occurred at amino acids 45 to 47 in V_λ and at 47 to 49 in V_κ in accord with the earlier suggestion (80) that CDR2 in V_λ consisted of residues 48 to 56 and in V_κ of 50 to 56; the match of seven nucleotides of κ and λ at residues 57 to 59, the junction of CDR2 and FR3, was back in register. The invariant Trp 35 in all V_L chains can also serve as a point of recombination since there is but one Trp codon.

In the absence of the V region nucleotide sequences this assortment would be consistent with a minigene hypothesis, but would involve a different recombinational mechanism from that established for the J and D minigenes. We suggested earlier (86, 88) that probes for the individual FR and CDR segments could serve to determine whether small segments of DNA existed separately from the assembled genomic V regions. This was considered a possibility because there are many more CDR than FR segments in all species and a cassette model might reconcile the assortment and the nucleotide sequence data (81). Moreover, all J and D clones isolated involved use of probes much larger than these segments themselves so that FR or CDR gene segments might have been missed.

Recently Komaromy and Wall (98) actually employed smaller probes and a more sensitive hybridization technique and could show assortment of FR segments at the DNA level; 32 clones containing only V_L regions were isolated by hybridization with a cDNA plasmid for the entire V region of MOPC21; eight hybridized to a probe coding for the precursor, the intervening sequence and FR1, residues 1 to 23; others hybridized to a probe comprising nucleotides of CDR1, FR2, and CDR2, residues 23 to 53, and still others to a probe of CDR2 and FR3, residues 54 to 83. Nucleotide sequencing may ultimately show whether all of these clones represent intact V regions (minus J).

The assortment data for the rest of the V region have also been considered by Egel (42) and by Baltimore (5) to arise by a gene conversion mechanism (see 149) in which conversion occurred by the recombinations at the junctions of the FR and CDR segments. This could account for the assortment data and would generate a large repertoire of distinct sites. It would also tend to account for idiotypic specificity associated with different antigenic determinants (18, 115, 116) since segments containing cross-reacting idiotypic determinants might as-

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sort with antibodies of different or distinct specificities (18, 116). It has been suggested in the $\alpha 1 \rightarrow 3$ antidextran system that the cross-reacting idiotype is associated with residues in CDR2 of V_H and the individual idiotypes are associated with the D segment (140). Although Baltimore (5) favors this gene conversion occurring in evolution, if such conversions were occurring somatically they could compromise the estimates of germ line V genes and perhaps contribute so substantially to diversity that it would eliminate the need to postulate random V_L-V_H association.

Another set of data that must be reconciled with the gene data is the finding of an identical FR2 segment, amino acid residues 35 to 49, in one human, 21 mouse, and 13 rabbit V regions (86, 87). Thus, this FR2 sequence has been preserved for about 80 million years. If intact V regions occurred in the germ line, one could count the number of copies of this preserved sequence from the sequence differences in the CDR1 and CDR2 segments on each side; such a count necessitated its presence in 5 of 6 BALB/c, 10 of 14 NZB, and 12 of 13 rabbit sequences. If differences in FR1 and FR3 were considered, there would have to be one copy for each chain. The question thus arises as to what is preserving this segment over evolutionary time. Table 1 shows (87) that it exists in the mouse and in the rabbit in many alternate forms that occur with relatively low frequency. Indeed, except for the Phe-Tyr substitution at position 36, the mouse and rabbit have entirely different substitutions thus indicating a largely separate evolution. FR2 is in an open portion of the Fab fragment and can accept side chain substitutions readily. Thus there is a hierarchy of use of FR2 segments, the preserved segment being used most of the time and the others, infrequently. Since they all constitute functional chains and if the preserved segment exists in so many copies in the mouse and in the rabbit, one wonders what is maintaining the hierarchy of use over 80 million years and why more of an equilibrium has not been reached.

There appears to be considerable belief that somatic mutation may be associated with the shift from IgM to IgG and contributing to the generation of diversity. Rodwell and Karush (130; see 91) propose from the similarity in isoelectric focusing patterns of horse antilactose antibodies that IgM patterns are highly restricted in the germ line. Gearhart et al. (50) found that the V_H regions of IgM hybridomas tended to be invariant whereas IgG_3, IgG_1, and IgA showed mutations in CDR1 and CDR2 but there were also a comparable number of mutations in the framework. Bothwell et al. (10) obtained cDNA clones

TABLE 1

*FR2 sets in mouse and rabbit V_κ chains and number of chains in each set including those found in human and rabbit V_κ**

Set	No. in Set	Residue														
		35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
1	1H, 20M 13R	TRP	TYR	GLN	GLN	LYS	PRO	GLY	GLN	PRO	PRO	LYS	LEU	LEU	ILE	TYR
2	6M 4R		PHE													
3	1M									SER						
4	1M												VAL			PHE
5	1M															LYS†
6	1M					ASN				SER						
7	1M							GLU		SER						
8	1M		LEU†			—‡	—	—	—	—	ILE		ARG			
9	1M			LEU		—	—	—	—	—	—	—	—			
10	3M		PHE						LYS	ALA						
11	1M							ASP	GLY†	THR	VAL†					
12	1M		PHE	LEU		ARG				SER		GLN				SER
13	4M						SER		THR†	SER			PRO	TRP		
1	1R												VAL			
2	1R												GLY			
3	1R						ALA						—	—	—	—
4	1R												ALA			
5	1R												GLY		LEU	
6	1R		PHE										ARG			
7	1R									ARG			VAL			
8	1R		PHE										GLY†			

* From E. A. Kabat, T. T. Wu, and H. Bilofsky, J. Exp. Med. **149:** 1299–1313, 1979.

† Two base changes. Amino acids listed in mouse sets 2–12 and rabbit sets 1–7 are those differing from set 1.

‡ —, Residues not sequenced.

Residues are identical to those in the top sequence except for substitutions listed.

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from two hybridomas making anti-NP^b (4-hydroxy-3-nitrophenyl acetyl) antibodies; these hybridoma antibodies had λ light chains and were heteroclitic reacting better with 5-iodo-NP than with NP. They also had a cross-reacting idiotype specific for IgG^b haplotype with one cDNA clone B1-8 making a μ and the other S43 a γ_{2a} heavy chain. Their nucleotide sequences in the V region differed at four positions in FR and four in CDR; their D segments varied substantially, but their J segments were identical. B1-8 and S43 resembled each other much more than five other cDNA clones that had been sequenced; of the sequences cloned they were most closely homologous to MPC-11. They both differed from all other V_H chains in having Pro at position 7.

Seven germ-line genes were sequenced one of which, 186-2, was identical in its coding sequence to B1-8. The seven genes fell into groups of three and four, respectively. All had Pro at position 7. The first group showed relatively few nucleotide substitutions none of which were in CDR1 and CDR2, whereas, of the second group of four, one had a two nucleotide substitution at amino acid 35 replacing His by Asp and all had differed extensively from group 1 in CDR2. One of group 1 had a replacement of Cys 22 by Arg that would result in a nonfunctional heavy chain since the domain disulfide loop formed by Cys22-Cys92 would be destroyed; a second genomic clone lacked a nucleotide coding for Cys 22 and thus would also be expected to be nonfunctional. It should be noted that if conversion of minigene Fr and CDR segments can occur it would be possible to rescue much of these two genes as well as the nonfunctional MOPC173B gene (109) having a mis-sense reading frame.

The shift from IgM to IgG is considered to be associated with increases in binding affinity and it will be of importance to compare binding constants of the various IgM and IgG hybridomas. In the α1→6 antidextrans studied by Sharon et al. (147), one IgM hybridoma had the highest association constant of any of the 12 hybridomas studied and a second fell in the middle of the IgA hybridomas.

Antigen binding variants arising in culture by somatic mutation of clones of S107 myeloma cells that bind phosphorylcholine have been studied by Cook et al. (27) and the sequence of the V_H regions determined by amino acid and by nucleic acid sequencing by Kwan et al. (101). One such mutant, V_H, which no longer bound phosphorylcholine, had a substitution of Ala for Glu at position 35 in CDR1; the sequence of residues 31 to 35, Phe-Try-Met-Glu, being found only in phosphorylcholine binding mouse myeloma proteins (84) and more recently in phosphorylcholine binding hybridomas* (50) with Glu35 having been shown by x-ray crystallography to be a contacting residue in the phosphorylcholine binding myeloma protein McPC603 (142); it is also significant that the only phosphorylcholine-binding human myeloma protein has the sequence Phe-Tyr-Met-Asp (128). The second variant, V_1, poses more of a problem; it had reduced capacity to hemagglutinate phosphorylcholine coupled-sheep erythrocytes and to bind phosphorylcholine coupled to KLH (keyhole limpet hemocyanin), but its K^a with phosphorylcholine did not differ from the parent S107. The only change in V_H sequence was the replacement of Asp 101 of CDR3 by Ala; residue 101, from the cloning studies, is also a part of the J minigene. Although position 101 is considered as a structural element in CDR3 (85) the substitution of the charged Asp by Ala might affect the site structure conformationally; how it could influence accessibility of phosphorylcholine-coupled proteins to the site without influencing the entry and binding of the small molecule is not clear.

Human V_κ genes have been isolated by hybridization with cloned mouse V_κ + C_κ cDNA (7). These also are contiguous through amino acid 95. Evidence for functional and nonfunctional (pseudogenes) V_κ and V_λ germ-line genes has been seen (7, 26), the latter containing termination codons and nucleotide deletions and insertions. Similar apparently nonfunctional genes in α and β globin have been reported. Another probably nonfunctional gene resulted from variation in the point of V-J joining (109); such aberrant rearrangements from V-J joining appear to be relatively frequent.

Of special interest are the findings that in B cell lines expressing κ chains, the λ genes remain in the germ-line configuration, whereas, in B cells that express λ chains, the κ constant region genes and the J minigenes are rearranged or deleted (2, 26, 59). It would seem, as Alt et al. (2) suggested, that appearance of a functional light chain stops additional light chain gene rearrangements and that differentiation may proceed sequentially from κ to λ.

There has been considerable uncertainty as to the origin of the immunoglobulin light and heavy domains. Hill et al. (61) suggested gene duplication from a primitive domain, but the differences between V and C domains made this acceptable only in a most general way. Recently, Williams and coworkers (14, 22, and in preparation) have sequenced the Thy-1 protein from mouse and from rat and found extensive homologies to both V and C domains that suggest that the primordial Thy-1 domain could be a precursor of both the V and C domains. It is of special interest that a homologized position 35 in V_L and 36 in V_H of Thy-1 contains Leu whereas all 231 V_L and 84 V_H chains contain an invariant Trp. Moreover, the MOPC173B (109) defective gene also has a Leu at position 35, as do all β_2 microglobulins (see 88). These findings should have important bearing on the origin of the antibody-forming system. The association of the Thy-1 antigen with T cells, the isolation of D-containing clones from T cells (100), and the demonstra-

* One phosphorylcholine hybridoma was reported (50) by amino acid sequencing to have the sequence Phe-Tye-Ile-Glu.

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tion of recombination within the J_H locus of T cells (28) are also relevant to the understanding of antibody specificity and complementarity.

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