GENERATION OF ANTIBODY DIVERSITY IN THE MEMORY RESPONSE TO PHOSPHOCHOLINE

by

Mary P. Stenzel-Poore

A Dissertation

Presented to the Department of Microbiology and Immunology and
the Oregon Health Sciences University
School of Medicine

in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

November 1986

Approved:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Figures (cont.)

ABBREVIATIONS

bp	base pair(s)
CDR	complementarity determining region
D	diversity region gene
DPPC	(N-(2,4 dinitrophenyl)-p-amino phenyl phosphorycholine
ELISA	enzyme linked immunosorbent assay
H	heavy chain
J	joining region gene
J_H	joining region gene - heavy chain
J_k	joining region gene - kappa chain
J_L	joining region gene - light chain
kbp	kilobase pair(s)
KLH	keyhole limpet hemocyanin
L	light chain
M141	MOPC 141 myeloma
M603	McPC603 myeloma
NPPC	nitrophenyl phosphocholine
OX	2-phenyl-5-oxazolone (phOX)
PC	phosphocholine
PJ14	MOPC 141 germline V_H gene
SP2/0-Ag14	nonsecretor myeloma
T15	TEPC 15 myeloma
V	variable region gene
Xid	X-linked immune deficient
V_H	variable region gene - heavy chain
V_L	variable region gene - light chain

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Marvin Rittenberg for his unfailing support and enthusiasm throughout this project and for encouraging me to achieve greater heights.

Special thanks to all those individuals in the lab whose humor and ideas were a valuable part of this project.

To my family who have always been a great source of happiness and encouragement to me.

Finally, I would like to express my deepest appreciation to my husband Peter who taught me the real meaning of collaboration and shared with me the excitement of these scientific endeavors.

STATEMENT OF PROBLEM

The immune response to phosphocholine (PC) is an attractive system for the study of the generation of antibody diversity. PC is a simple, chemically-defined hapten which exists as a common environmental antigen in a variety of microorganisms such as Streptococcus, Morganella, Aspergillus and Ascaris (1-3). The antibody response to PC has been the focus of intense study providing a useful system to analyze the mechanism of idiotype selection and regulation, the genetic basis of antibody recognition and mechanisms of clonal dominance (reviewed in 4-6). The response to PC is a particularly useful model of immune maturation and the development of antibody diversity because it initiates as a contracted, highly focused antibody response that evolves into a heterogeneous response, one that exhibits a new binding phenotype and idiotype(s) distinct from those antibodies found during the primary response (7-9). The basis of the evolution of heterogeneity in the PC memory response is the major focus of this research project.

B-cell participation during the initial response to PC in Balb/c mice is primarily limited to those clones expressing the product of a single V_H (V_H1) and V_k gene (V_k 22) (10,11). The majority of these antibodies bear idiotypic determinants related to those present on a prototype PC-binding myeloma protein TEPC15 (T15) (12,13). Thus, the primary response to PC is restricted in gene usage and idiotype expression, and the binding specificity of these antibodies is homogeneous exhibiting appreciable affinity for both PC and nitrophenyl-PC (NPPC); we have termed these antibodies Group I (8).

In contrast to the pauci-clonal nature of the primary response, the memory response is heterogeneous, exhibiting in addition to Group I antibodies, a new family of antibodies that differ from Group I in fine specificity and idiotype expression. These new antibodies, which we have termed Group II lack the T15 idiotype and have appreciable affinity for NPPC yet bind PC only weakly (8,14). The origin of these antibodies could be accomplished through several processes. Existing clonotypes could be modified by somatic mutation of variable genes (V) to yield changes in antibody specificity. Molecular heterogeneity could also develop through the use of new V genes or V gene combinations that result in new and distinct antibody specificities. B cells expressing the new or modified V genes could then be recruited and expanded as the antigen-driven response matures.

The theme of this project was to study the molecular basis for antibody heterogeneity, in particular Group II antibodies in immunological memory to PC. The hypothesis to be tested was that the primary basis for this heterogeneity was due to recruitment of additional V genes rather than somatic mutations in V genes prevalent in the primary response.

The objectives of this thesis were:

1. To identify the V_H gene utilized by a representative Group II antibody (non-T15-like) from the memory anti-PC response by:
   a) cloning and sequencing the V_H gene expressed by a prototype Group II hybridoma (referred to as PCG1-1).
   b) sequence comparison to known V_H sequences to obtain an assignment to a specific germline V_H gene and/or V_H gene family, and

c) establishing the unique identity of this V_H gene by Southern blot hybridization using a 5' V_H flanking probe.

3. To determine the contribution the PCG1-1 V_H gene makes to the Group II antibody response by generating Group II-like hybridomas that can be analyzed for V_H expression utilizing Southern and Northern blot hybridization.

4. To determine the extent of V_H heterogeneity in Group II antibodies by:

   a) examining J_H rearrangements using Southern blot analysis and

   b) analyzing whether the basis of T15 idiotype negative antibody formation in selected hybridomas results from the use of gene segments distinct from V_H1.

While probing the genome of a cell line unusual structures are sometimes revealed that may provide insight into normal cellular mechanisms through their abberrant nature. In the course of cloning a rearranged V_H gene, a novel immunoglobulin rearrangement was identified that led to DNA sequence analysis in order that the detailed structure of the rearrangement could be determined and to Southern blot experiments to determine whether the unusual immunoglobulin gene segment that was involved in this rearrangement pre-existed in the germline or was merely an aberration confined to the B cell from which the hybridoma had been made. These questions in addition to the objectives outlined above were addressed in this thesis project.

INTRODUCTION AND LITERATURE REVIEW

General Features of the Anti-Phosphocholine Response

Antibodies to PC serve as an example of a restricted response whose B cell repertoire appears to be small (15) and highly conserved (16). The dominant fraction (which may represent as much as 99%) of anti-PC antibodies in BALB/c mice possess idiotypic determinants found on the prototype PC-binding myeloma TEPC (T15) (12). The remaining fraction comprised a set of anti-PC antibodies lacking the T15 idiotype ($T15^-$) and which share idiotypic determinants with the PC-binding myelomas McPC603 (M603), MOPC 511 (M511) and MOPC 167 (M167) (6). Amino acid and nucleotide sequencing of heavy (H) and light (L) chains revealed that distinct V_L regions were used, V_k22 for members of the T15 group, V_k8 for M603 and V_k24 for M511 and M167; however it appeared that only one V_H region-V_H1 (also referred to as V_H4) was utilized in association with these three L chains (11,17,18). Thus, it was suggested by these data that V gene usage among anti-PC antibodies was restricted to a single V_H and three V_L segments. This view, as will be discussed later, is no longer accurate. The hapten binding profiles of antibodies bearing distinct L chains differ in their relative abilities to bind choline analogs (19) suggesting that fine specificity differences may be associated in part with light chain expression. Nevertheless antibodies from all three families display appreciable affinity for the haptens PC (18) and the nitrophenyl PC derivative (NPPC) (hapten structures, Fig. 1) (8).

In contrast to the earlier findings, Chang et al., described the appearance of anti-PC antibodies that lacked the T15 idiotype and were able to bind well to NPPC but only weakly to PC (8). Although these antibodies are virtually absent in the primary response, they represent as much as 50% of the anti-PC antibodies seen in the memory response to PC coupled to keyhole limpet hemocyanin (PC-KLH) (antigen structure shown in Fig. 1). In addition, these new antibodies (referred to as Group II) dominate the IgG_1 and IgG_2 isotypes whereas T15-like antibodies (Group I) are predominantly found in IgM, IgA and IgG_3 antibodies. It is possible that Group II antibodies were previously not observed due to their late appearance in the response (9), and the fact that earlier studies often used the whole bacterium Streptococcus pneumoniae (R36A) as the immunogen which is only known to induce Group I antibodies (16,20). More importantly, Group II antibodies may be easily overlooked during purification since these antibodies are specifically eluted from PC-sepharose using NPPC in contrast to Group I antibodies which can be eluted with both PC and NPPC. Traditionally anti-PC antibodies have been purified using PC as the eluting hapten (16) which could explain the apparent absence of Group II antibodies prior to their recognition in these studies which employed an NPPC elution step following PC elution of Group I antibodies (8).

While the T15 clonotype is highly represented in the anti-PC response in the majority of mouse strains (16), the immune defective CBA/N strain stands out as a notable exception to this observation (21,22). The X-linked immune deficiency (Xid) of this animal manifests itself in a number of immune abnormalities (23) which stem from a defect in B cell maturation. These mice have a selective inability to produce

antibodies to soluble polysaccharide antigens. They are unusual in that they are unable to respond to certain thymus independent forms of PC antigen and manifest a response to PC that is lacking in the T15 idiotype following immunization with a thymus dependent (TD) PC antigen such as PC-KLH (21). (CBA/N x BALB/c) F_1 animals provide a useful means of investigating Xid antibody responses since the recessive gene is expressed only in the hemizygous or homozygous state thus affecting only the males and leaving the female littermates phenotypically normal.

Recent studies indicate that Xid mice 1) respond to TD forms of PC but that they fail to produce IgM anti-PC antibodies (24), 2) produce normal levels of IgG anti-PC in a secondary response (22), and 3) exhibit little or no $T15^+$ antibody in their primary or secondary serum (21,22). Although Xid mice fail to produce a significant T15 response, they have been shown to contain normal numbers of T15 precursors ($T15^+$, anti-PC) in the bone marrow (25). Despite the presence of such precursors, Xid mice fail to produce a T15 response and instead are found to express anti-PC antibodies that are phenotypically indistinguishable from Group II antibodies (26). Although negative for the T15 idiotype, Xid B cells can produce antibody possessing V_H determinants common to members of the T15 family (24,27,28). Furthermore, an anti-PC hybridoma from a CBA/N mouse was found to have rearranged the V_H1 allele (29). In this hybridoma, the V_H1 gene was associated with an unusual D region and J_H segment as well as an atypical light chain, V_k3. The antibody was not considered to be Group II-like because although it bound PC poorly, it exhibited higher affinity for PC than for NPPC. These data indicate that the Xid defect does not preclude the expression of the V_H1 gene although there is evidence that the entire V_H gene family is expressed at

abnormally low levels in pre-immune Xid B cells (30). Thus it became of interest to determine whether the Group II antibodies made by the F_1 males were $T15^-$ due to the use of V_H genes other than V_H1 or to the expression of different D (and perhaps J_H) segments that would lead to $T15^-$ antibody formation. These studies are described in Paper 1 of this thesis.

The discovery of Group II antibodies suggested that the complexity of the anti-PC response was much greater than originally proposed. To determine the molecular basis for the origin of these antibodies, hybridomas were generated from mice undergoing a memory response to PC. Amino acid sequence analysis of several hybridomas that represented the Group II anti-PC phenotype ($T15^-$, bind only NPPC but not PC) revealed the use of a novel L chain (V_k1-3) not observed in the PC response (31) in association with a V_H gene whose NH_2 terminus was blocked and thereby inaccessible to Edman degradation (14); the latter was also a novel finding for anti-PC antibodies.

These findings indicated that the use of new V_H and V_L genes could explain the increased phenotypic heterogeneity that developed in the memory response. Although it is formally possible that the blocked NH_2 terminus represented a mutation of the V_H1 gene normally associated with Group I antibodies, it seemed unlikely at the time since partial amino acid sequence analysis of 42 anti-PC heavy chain proteins had not identified any with a blocked NH_2 terminus (32). In addition, Southern blot analysis of a Group II hybridoma with a blocked NH_2 terminus demonstrated that the V_H1 gene was not rearranged, further indicating that a distinct V_H gene was likely to be utilized by this hybridoma (14).

Molecular Properties of Anti-PC Antibodies

The formation of a functional V region requires the rearrangement of three sets of H chain (V_H, D and J_H) and two sets of light chain (V_L and J_L) germline gene elements (33). Structural diversity can be accomplished in several different ways. Germline diversity is obtained from the presence of multiple gene segments. For example the mouse H chain can be formed from any (presumably) of 100-300 V_H genes (34) ~12 D segments (35) and 4 J_H elements (36). Murine L chains exhibit a similar degree of germline diversity having >100 V_k genes (37) and 4 functional J_k segments (38). In contrast the murine λ chain must derive diversity from only two V_λ genes that can join with three J_λ segments (39). Additional variation is due to combinatorial diversity resulting from the use of different combinations of V, D and J and different pairing between H and L chains (33). Both H and L chain formation derive substantial diversity at the junctional boundaries of the individual gene segment due to the imprecise nature of the rearrangement process (40,41).

Superimposed upon the pre-existing germline diversity is the effect of somatic mutation which can cause amino acid substitutions throughout the V region thus increasing the available antibody repertoire (33).

Germline Diversity in Anti-PC Antibodies

The pattern of genetic diversity that emerges regarding Group I (T15-like) antibodies is one of restricted gene usage drawing from a single V_H gene and three V_L segments. V_H1 is observed in nearly all Group I antibodies. This is interesting since V_H1 derives from a small V_H gene family (S107) containing four members related to one another by greater than 85% sequence homology (11). Despite this high degree of

homology only one of the other members (V_H11) has been observed in anti-PC antibodies (32,42). Of the two remaining members, one is a pseudogene which contains two in phase termination codons (43) while the other (V_H13) appears functional by sequence analysis (11). It has been proposed that since mice bearing antibodies of the T15 idiotype are highly protected against in vivo challenge with viable pneumococci (44) there may be strong selective pressure to maintain this gene (V_H1) in the germline (32). Anti-PC binding antibodies from widely separate species share extensive sequence homology with V_H1 further supporting this view (45). Perlmutter et al. have suggested that frequently encountered antigens (such as PC) would impose a strong selective pressure on the evolution of the germline repertoire such that the effects of somatic mutation and antigen selection would best operate on genes which encode proteins with high affinity for the antigen (46). Thus, the entire germline V gene composition would be fixed in response to significant pathogens in the surrounding environment (46). Interestingly, all of the Group I-like BALB/c anti-PC V_H regions utilize the same germline J_H segment-J_H1 (32). It has been proposed that such restricted J_H usage reflects a role for this segment in antigen binding. The finding that a tissue culture derived mutant of the myeloma S107 (T15) selected for loss of binding to PC-KLH has a single replacement substitution at position 113 in the J_H segment (47) is consistent with the view that restricted J_H segment usage may reflect strong selection by antigen. While there is an apparent preference for J_H1 usage in anti-PC antibodies, the requirement does not appear to be absolute. Three anti-PC antibodies have been isolated that utilize different J_H segments. One hybridoma, generated from the immune defective mouse strain CBA/N, has been shown to use the

V_H1 allele-C3 in association with J_H4; the anti-PC antibody is also unusual in the use of the V_k3 gene product. Even though this antibody was shown to have a lower affinity for PC when compared with T15, it demonstrated a markedly higher affinity for PC than NPPC, thus indicating that it was not a Group II antibody. Two anti-PC hybridomas have been observed that use the J_H3 segment in association with V_H genes other than V_H1; one originated from a CBA/J mouse and was reported to use the V_H11 gene which is > 85% homologous with V_H1 and belongs to the same V_H family (S107) (42,48). In our laboratory we have generated a BALB/c hybridoma with Group II anti-PC binding characteristics that uses the V_H141 gene from the Q52 V_H family joined to J_H3 (discussed in Paper 2). It is clear from these data that there is not a strict requirement for J_H1 usage in PC binding antibodies. It is possible that in addition to selection by antigen there may be preferential V_H-J_H associations at the molecular level which play an active role in determining the composition of anti-PC antibodies.

The D segment employed by anti-PC antibodies appears to be restricted in length - the majority consist of five amino acids (49). A minimum of four different germline D segments have been described that draw from all three D families in the formation of PC-binding heavy chains (49). The majority employ the DFL16.1 gene segment wich encodes the amino acid sequence Tyr.Tyr.Gly.Ser.Ser. D gene segments within the SP2.3 gene family also contribute to the diversity of anti-PC antibodies and encode the related sequence Tyr.Tyr.Gly.Tyr.Asp. and one antibody has been found to use the Q52 segment (42). Sequence comparison of D segments used by PC-binding antibodies from different mouse strains (BALB/c and C57BL) indicate that they may use the same genes while

proteins from the same strain may use different D regions (50). Moreover, despite a seemingly wide variation in D gene usage, these proteins exhibit remarkedly similar association constants for PC hapten. D region expression does not appear to correlate with the form of antigen used - e.g., PC-KLH and Proteus morganii both induced antibodies with identical D regions. Conversely, a given antigen can induce antibodies with different D segments (50). Thus, the role of D segment expression in hapten binding may be minor although it is possible that the length of D may be important in determining the tertiary structure of the antibody combining site. D segment usage appears to play a major role in T15 idiotype expression since it has been shown that $T15^+$ antibodies in BALB/c mice utilize the V_H1 gene joined to DFL16.1 and J_H1 (in association with V_k22) and that a single amino acid difference resulting in a D region longer by one amino acid results in the loss of the T15 idiotype (51). Additional support for the notion that the D region plays a major role in determining the T15 idiotype lies in the fact that A/J mice known to produce very little $T15^+$ antibody in response to PC may express the identical V_H, V_L and J_L gene segments as those found in $T15^+$ antibodies but differ sharply in D region expression (52). It was proposed from these data that A/J mice may lack the germline DFL16.1 gene and therefore only those antibodies which had somatically generated a D region resembling the BALB/c DFL16.1 gene would yield the T15 idiotype.

It is clear from the previous discussion that the PC system exhibits very little genetic diversity in V_H and J_H gene selection; however diversity is contributed through selection of different D region elements. Additional diversity may derive from alternative recombination sites at the V_H-D and D-J_H splice points resulting in the generation of

deletions, insertions and hybrid codons at the junctional boundaries (32). Some anti-PC antibodies contain insertions at these junctions that cannot be attributed to germline V_H, D, or J_H segments and are presumed to be the result of de novo addition of nucleotides to the 5' or 3' boundary of D segments (54).

As mentioned above light chain expression in Group I anti-PC antibodies draws from three major families of V_L genes V_k8, V_k22, V_k24, and would thus appear to be less restricted genetically than H chains in forming PC binding sites. Complete sequence analyses of the V_L genes from T15 (V_k22), M603 (V_k8) and M167 (V_k24) (55,56,57) have demonstrated that they differ widely in both framework and hypervariable regions. Furthermore, proteins expressing these three light chains in combination with the V_H1 gene product have different, characteristic specificity profiles for a series of choline analogs (19,58). Each family of antibodies was found to display its own unique binding profile using hapten inhibition of binding to PC linked to different carriers. Such antigen binding differences have been taken to implicate a role for V_L in fine specificity determination even though the bulk of the hapten-antibody interaction is thought to involve contacts with V_H regions (50). It is noteworthy that while the prototype anti-PC V_L genes discussed above exhibit striking sequence differences, all three light chains have the amino acid sequence Tyr.Pro.Leu at positions 94, 95, and 96. This is a feature unique to anti-PC antibodies; no other antibody has been found to contain this sequence at this position (59). It has been shown by X-ray diffraction analysis that Tyr94L of the L chain hydrogen bonds with the V_H residue Glu35 and may also form a hapten contact (60). Leu96L which is derived from the J_k segment also contacts

the hapten (57). Thus, the amino acids in the V_L third hypervariable region serve an important function in generating the PC binding site. It is evident that, although three very distinct L chains contribute to Group I antibodies a strong selection process exists that guides the use of V_L genes that share what appears to be a required structure. The V_L genes found in Group I anti-PC antibodies have been shown to be restricted in J_k selection utilizing J_k5 in all cases described (32,55,61) including the prototype antibodies from T15, M603, M511 and M167 (50,59). The use of J_k5 correlates directly with antigen binding. X-ray crystallographic examination of the M603 binding site reveals that Leu96L encoded by the first amino acid of the J_k5 segment (38) forms a hapten contact (57).

We have found that Group II antibodies exhibit even greater L chain diversity than Group I antibodies. Anti-PC antibodies from the Group II response were shown to utilize the V_L genes V_k1-3, and $V_\lambda2$ not previously observed in Group I antibodies as well as those V_L genes that are represented in Group I antibodies (31,62). More recently, the $V_\lambda1$ gene product has also been shown to contribute to Group II antibodies thus extending the range of V_L elements observed in the PC immune response (Paper 2 and ref. 63).

Somatic Mutation in Anti-PC Antibodies

The repertoire of antibodies which is produced by an individual can be attributed to two genetic origins: germline and somatic. As discussed above, germline diversity is derived from numerous gene segments, random pairing of these segments, imprecise joining of these elements and

combinatorial association of heavy and light chains, e.g. V_H1-V_k22, V_H1-V_k24 etc. The germline repertoire is expressed by primary B cells and is most often seen in the initial phase of an antigen driven immune response. It has been observed that IgM antibodies generally represent the early response and that their V regions often reflect germline sequences (18,64,65). Antigenic stimulation of primary B cells results in selective clonal expansion which is accompanied by a high rate of somatic mutation - a frequency estimated to be 10^{-3}/base pair/generation (66,67). Evidence is accumulating that somatic mutation is randomly distributed in and around V genes (61,68), as single point mutations (33), and is localized to the V gene segment, (61,69), although it is difficult to assess these features accurately since only a few mutations are generally observed in a particular V_H gene and most often only a small number of somatically mutated V gene sequences can be compared to a single germline V gene. It has been proposed that the process of somatic mutation is linked to the immunoglobulin class switch due to the finding that non-IgM antibodies frequently bear mutations whereas no mutations have been observed in IgM antibodies expressing the same V gene (18,64,70). While there may be a higher frequency of somatic mutation in antibodies that have undergone a class switch, it is clear that class switching is not required for activation of the somatic mutation mechanism since there is evidence for somatic mutation in IgM producing B cells (71,72). It appears that the process of somatic mutation may be more directly linked to cell division or DNA synthesis (73) perhaps via a mechanism of error-prone DNA repair (61).

Somatic mutations of anti-PC V_H and V_L genes contributes substantially to their genetic diversity (18,32). Gearheart *et al.*

found no diversity in the V_H segments from 13 IgM hybridomas when sequenced through the N-terminal 36 amino acid residues (18,74). In contrast, 11 of the 28 non-IgM antibodies had amino acid substitutions within the first 36 residues which led to the hypothesis that the mutational mechanism is activated in B cells after they have switched from IgM. Somatic mutation of V_L regions was also found to correlate with the class of antibody produced: 10 out of 25 V_L chains from IgG or IgA antibodies were mutated whereas only 1 of 14 IgM antibodies contained a mutation (74). The fact that V_k22 derives from a multi-gene family (37) has made it difficult to determine whether the observed sequence variations in V_k22 anti-PC antibodies represent somatic mutations of a single gene or if they represent expression of closely related family members that exhibit only minor differences from one another in the germline. V_k8 also originates from a multi-gene family (37) and sequence comparisons have been complicated by the same difficulty as seen in V_k22 sequences. Although V_k24 shares homology with a family of 7-10 members (75) a single germline gene appears to be most homologous with the V_k24 anti-PC antibody and thus has been considered to be the primary gene from which somatic variants arise (76,77). Four different V_k24 proteins from this gene have already been identified (61) which indicates these have arisen as somatic variants of the V_k24 germline gene. It is clear that the germline sequence encodes a light chain that can be used to bind PC since two hybridomas express the germline product and bind DPPC with affinities comparable to $T15^+$ antibodies indicating that somatic mutation of V_k24 is not required to generate an adequate L chain.

Somatic mutations introduce diversity into responses to other antigens such as 2-phenyloxazolone (OX) (72,78), p-azophenylarsonate

(ARS) (79,80), influenza virus hemagglutinin (HA), (66,68,81) and (4-hydroxy-3-nitrophenyl) acetyl (NP) (64). The overall effect of somatic mutation on the immune response is thought to increase the relative affinity of the participating antibodies. Repeated rounds of antigenic stimulation caused by low levels of antigen may selectively expand those antibodies that have acquired better fitting combining sites via somatic mutation. The acquisition of higher affinity combining sites may confer a proliferative advantage upon somatic variants which would drive the response to mature with a net high affinity character. Although the notion that somatic variants would have higher affinity combining sites than their germline counterparts is intuitively satisfying, until recently there has been very little data to support this idea. However, the OX response provides a good example where antibodies obtained very late in the primary response contain mutations in their V_L and V_H regions and have higher affinities for the OX hapten (2-10 fold) than unmutated antibodies (72). It was proposed from these data that following an initial dose of antigen that diminishes slowly over time those clones bearing higher affinity receptors (via somatic mutation) would be preferentially expanded and observed later in the response.

Affinity maturation in the PC system has not provided such a clear picture. Somatic mutation has been firmly established in anti-PC antibodies as discussed above. Initial affinity measurements using PC as the hapten compared the relative affinity of IgM (representing unmutated V genes) and IgG antibodies as reflected in hybridomas (18). The findings indicated that in spite of a prolonged period of immunization the IgG antibodies did not display a maximum affinity greater than that

of the IgM antibodies. It was subsequently proposed and tested that the binding constants obtained with the simple ligand PC might not constitute an adequate measure of the affinity of the antibodies for the complete antigen (82). Furthermore, they reasoned that since the immunizing agent was prepared by the conjugation of p-azophenyl PC to a carrier protein the added presence of a hydrophobic phenyl group could contribute substantially to the binding constant and thus allow a distinction to be made between IgG and IgM anti-PC antibodies. Using the phenyl PC fluorescent compound, (N-2, 4-dinitrophenyl)-p-aminophenyl-PC (DPPC), these authors observed that the presence of a phenyl group markedly altered the binding constants (compared with PC alone) but only in the IgG antibodies. Second, the maximum affinity of the IgG group of hybridomas was not attained by the IgM group. These data indicate the importance of selecting the appropriate ligand to determine the relative binding constants and more importantly suggest that the effects of somatic mutation may be masked unless the ligand closely resembles the complete antigen that was used to expand high affinity variants selectively in the course of immune maturation.

The nature and location of somatic mutation in expressed V genes is important in determining the specificity of antibody. The effects of this process can diminish or destroy as well as enhance the ability to bind antigen. While anti-PC antibodies containing multiple mutations have been found with unchanged or increased affinity (18,82), single mutations within the V-D-J region have been found that result in complete loss of (83) or diminished binding (47). These mutants have been used to determine the relative importance of specific amino acid residues. Two anti-PC variants have been described that have lost the ability to bind

PC or PC-protein due to a single amino acid substitution in the H chain. Both variants were derived by somatic mutation in tissue culture from the S107 myeloma cell line (84); in one case (S107.U_4) the mutation was in the first hypervariable region resulting in a Glu to Ala interchange at position 35 and the other variant (S107.U_1) had a single change in the J_H-1 segment resulting in a substitution of an Ala for an Asp. The Glu to Ala substitution at position 35 in S107.U_4 resulted in complete loss of binding to PC-protein. In M603 Glu35H was originally designated as a hapten contact residue (85), but based on refined X-ray structural analysis the distance between Glu35H and the PC hapten makes its role in direct hapten contact somewhat uncertain (50,57). However, structural analysis does indicate that the side chain of Glu35H forms a hydrogen bond with the phenolic hydroxyl of the light chain residue Tyr94. This interaction may serve to stabilize the binding pocket and thus substitution of an Ala in this position might destroy the integrity of the hapten binding site. It was reasoned from these data that if in fact Glu35H were only a hapten contact residue it might be expected that a substitution to the smaller alanine side chain would only diminish binding but not destroy it (83). Additional support for this lies in the observation that V_H11 encodes a protein highly homologous to V_H1 (expressed by S107) but to date has been observed in only two anti-PC hybridomas. Although most of the hapten contacting residues are present, position 35H of the germline V_H11 is a serine instead of glutamine as it is in V_H1. The effect of this difference may only be to lower the affinity of PC binding since it was shown that one of these hybridomas had the lowest association constant for PC of all hybridomas tested (18). Thus serine at 35H of V_H11 may maintain enough of the needed conformation

to permit minimal binding to PC. It is interesting that while S107.U_4 lost PC binding it appeared to have acquired new antigen reactivity in that it bound double-stranded DNA as well as the phosphorylated molecules, cardiolipin and protamine (86). This finding would seem to indicate that a germline gene that does not encode an autoantibody can as a result of somatic mutation produce an autoantibody. Thus a germline gene that is amplified in response to a bacterial antigen can produce clonal progeny that will react with self-antigens. S107.U_4 has been observed only in vitro and thus it is not known whether such an antibody could be induced and cause disease in vivo.

In contrast to the observations with S107.U_4, the finding that S107.U_1 has lost the ability to bind PC-protein as the result of a single change in the J_H region is somewhat curious because there is no evidence that this region is directly involved in binding. Due to the fact that binding of the free PC hapten was unaltered in this mutant while reactivity to hapten-carrier conjugates was diminished, it was proposed that the J_H region substitution produced a change in the configuration of that portion of the combining site that interacts with a variety of carriers (47).

The results obtained from the S107 binding mutants demonstrate that small numbers of changes can produce profound alterations in antigen binding specificities. Although it is obvious from analyses of many anti-PC antibodies that substitutions generally do not result in such drastic changes (18) in anti-PC reactivity, antigen binding loss variants would be overlooked in most selection procedures. It is also clear that antibodies expressing the germline V_H1 gene bind PC with relatively high affinity thus indicating that somatic mutation is not a

requirement to generate an adequate anti-PC combining site. It is likely that it may serve to diversify the anti-PC population from which clones may be selectively expanded by antigen thus shaping the overall character of the antibody response as it matures.

Three Dimensional Structure

The nature of the antibody combining site and its interaction with antigen is a function of its amino acid sequence. The recent application of X-ray diffraction to immunoglobulin fragments has lead to an increased understanding of the nature of antigen-antibody contacts and H and L chains interactions (60,84,85,88-90). The three dimensional structure for the Fab of M603 with PC in the combining site has been resolved to the level of 3.1-Å (85) and, in combination with an extensive collection of amino acid sequences from anti-PC antibodies, has enabled the assignment of critical residues necessary in forming an anti-PC combining site.

The site of hapten binding is in a large cavity which measures 12-Å deep, 15-Å wide and 20-Å long (85). The cavity is lined by all three of the heavy chain hypervariable regions H1, H2, and H3, and two of the L chain hypervariable regions, L1 and L3. PC occupies only a small part of the cavity and is bound asymmetrically, being located closer to the H chain. The choline moiety is buried deep in the cavity and the phosphate group extends towards the exterior. The positively charged trimethylammonium group of the hapten interacts with the acidic residues Glu58H and Asp91L; and may contact a more distant Glu35H which is located 5-Å away from the charged nitrogen (85) although it has been suggested

that this distance may be too great to allow a direct contact (57). Leu96L, the first amino acid of the J segment is involved in hapten contact at the back of the binding pocket (57). In addition, the L chain residues 92-94 are close enough to the choline to provide van der Waals contacts and it has been suggested that Tyr94L is extremely important to the structure of the binding site by forming a hydrogen bond with Glu35H (57). The phosphate group appears to interact exclusively with heavy chain amino acids. The subsite for phosphate binding contains Tyr33H, Lys52bH and Arg52H which serve as hydrogen bond donors that contact the phosphate oxygen (57).

Comparison of the amino acid sequences of anti-PC antibodies has been useful in determining the degree of conservation between proteins and in particular, those amino acids implicated in binding (91). There is extreme conservation of those residues that form contacts between H and L chains as well as those involved directly in hapten binding. Although these studies have taken into account antibodies that have Group I-like binding features similar to that of M603 they may also be useful in making inferences about the hapten binding characteristics seen in Group II antibodies as those sequences become known (see Paper 2).

Somatic Evolution of the Immune Response

B-cell clones are selected for participation in the immune response via interactions involving their surface immunoglobulin. Binding of antigen to these receptor antibodies constitutes part of a signal that induces proliferation, differentation, antibody synthesis and secretion. It is well known that the phenotypic character of the antibody response

to most antigens changes as the response progresses (92,93). The basis for these changes has become an area of intense interest since the development of the invaluable technique of clonal immortalization by generating B-cell hybridomas (94). The ability to isolate individual clones participating in a response to antigen by fusing splenic B cells with myeloma cells has revolutionized immunoglobulin V region analysis by providing unlimited supplies of antibody and nucleic acids with which to study binding specificity and molecular structure. It has made possible examination of the temporal composition of antibody responses at the clonal level which has given insight into the mechanism(s) responsible for clonal selection and dominance (72,78,95-98). The general theme of immune maturation appears to depend upon the selection of particular gene segments as well as the selection of structural variants that represent somatic mutants (99). The evolution of a response is driven by selection in the form of antigen (92,100) and/or idiotype regulation (101,102). Changes in Ig receptors of individual clones would alter the degree of participation of such clones during the evolution of the antigen driven response and thus modify the composition of the expressed repertoire.

The population dynamics of the temporal response to p-azophenylarsonate (ARS) (95,103) and 2-phenyloxazolone (OX) (72,78,96) have been recently investigated and the findings have lead to the development of an elegant model concerning the generation of functional immunity (99). While the basis for this model is derived from a response (ARS) that has some differences from the PC system, it will be considered here as a progression of events that concerns general antibody development and specifically how it may apply in the immune response to PC. The structure of this model involves four main stages that in all

likelihood are a continuum of genetic processes in response to selective forces. The four stages are: 1) Stage 0 - The preimmune repertoire, 2) Stage 1 - Clonal selection, Part 1, 3) Stage 2 - Variations of combinations and somatic mutation and 4) Stage 3 - Clonal selection, Part 2.

Stage 0 - The preimmune repertoire. Spleen cells from non-immune mice represent a very diverse B-cell population. It has been estimated that the IgM repertoire exceeds 10^7 clonotypes (104) and that any one clonotype represents a small fraction of the total population (75,77,104-106). Thus, in the case of the BALB/c anti-PC response, 1 in 10^5 B-cells yielded anti-PC antibody that was indistinguishable from T15. An additional 1 in 10^5 yielded non-T15 anti-PC antibodies. Fine specificity and isoelectric focusing analysis indicate that the number of different anti-PC clonotypes generated in the preimmune animal is greater than 10^2 (104). These studies employed the splenic fragment assay (107) which is based on the principal that spleen cells removed from an animal and transferred into an irradiated recipient will repopulate the host spleen. The spleen can then be removed and divided into fragments that may be cultured in vitro in the presence of antigen. B-cells capable of responding to antigen proliferate forming foci of cells that are monoclonal in origin, and the antibodies from these clones may be examined for fine specificity and idiotype. While these studies may assess the frequency of PC reactive clones it is possible that they represent an incomplete picture of the true repertoire that is capable of binding antigen. It is not clear whether there may be B cells that actually bind antigen and yet are not triggered to divide and expand in which case they would remain undetected. The use of the B cell mitogen,

lipopolysaccharide (LPS) (108) as a means of stimulating cultures circumvents the requirement for antigen stimulation and relies on the mitogen to activate the cells independent of their antigen receptor. Several reports using LPS have measured the frequency of anti-PC clones and found 5-50 fold more precursors in the adult spleen than were detected using PC-KLH in the splenic fragment assay (109,111). Despite the differences in absolute frequency, in most cases the T15 clonotype represented approximately one half of the total PC-binding clones. This finding has been extended to include B cells that lack surface Ig receptors and originate in the bone marrow, which has been interpreted to mean that T15 dominance occurs at the prereceptor level and therefore results from factors other than environmental selection by antigen (25).

The main difficulty with these studies is that they do not allow the assessment of the molecular diversity of the V region structure expressed by preimmune B-cells. The studies discussed above address only the phenotypic frequency of anti-PC precursors, the actual frequency of T15 V_H1 has not yet been determined. The recent development of a method designed to analyze the expressed V region of hybridomas by in situ hybridization of V gene specific probes to cellular mRNA has extended the range of information to include the frequency of specific V gene expression and V_H and V_L associations (103,112). These investigators analyzed the frequency of the V_H gene encoding a major cross-reactive (Id^{CRI}) idiotype in the anti-Ars response and found that 1 in 400 B cells expressed the gene (103). This value is consistent with the idea that the gene is randomly expressed in the total repertoire. More importantly, nucleotide sequence analysis revealed that the V_H gene element randomly associated with D, J_H, V_L and J_L segments suggesting

that the preimmune repertoire develops in a stochastic manner. Thus the restricted V_H gene associations observed in the anti-ARS system (and by analogy, anti-PC) may result from selective forces that act during and not prior to the immune response.

Stage 1: Clonal selection, part 1. Immunization results in stimulation of a unique subset of B-cell clones that exist in the preimmune population. The initial phase of the immune response to PC is characterized by antibodies that are restricted in variable region usage, predominantly of the IgM isotype and without somatic mutation (18,20). The overwhelming majority of primary antibody is Group I and $T15^+$. The reason for such homogeneity in V region expression is an unresolved issue since as discussed above, it is clear that the preimmune repertoire contains a substantial $T15^-$ PC binding subset. Different activation requirements may bias the expression of certain clonotypes and could explain the restricted antibody pattern seen during the primary reponse (27,113).

It appears that environmental factors such as exposure to normal food antigens and living organisms do not impose a predilection for T15 dominance since BALB/c mice raised on synthetic diets in a germ free environment exhibit a T15 dominant response following PC immunization (114). The inherent difficulty in this kind of experiment is the development of a completely germ free environment since PC-containing antigens are ubiquitous being present even in host tissue. The same authors have proposed that the increase of $T15^+$ clones from the preimmune frequency of 50% to greater than 95% of the response following primary immunization is due to intrinsic fine specificity differences in the

antigen receptors of $T15^+$ vs. $T15^-$ B cells (115). The basis for this proposal lies in their observation that the $T15^+$ B cell response has a 7-fold higher avidity for PC than $T15^-$ B cells in BALB/c mice. Thus an important component of clonal dominance is the affinity of antigen receptors on antigen sensitve cells. Further support for this is the finding that mouse strains that do not characteristically exhibit T15 dominance but rather portray a more equal distribution of $T15^+$ and $T15^-$ PC antibodies in the primary response exhibit equal avidities for PC in both populations (115). These studies analyzed only primary IgM antibodies ($T15^+$ and $T15^-$) that were inhibitable by free PC which would preclude Group II antibodies from analysis should they have been represented in the $T15^-$ population. It is very likely that the $T15^-$ antibodies observed in a primary response represent M603 and M511/167-like antibodies that express the V_H1 gene in combination with V_k24 and V_k8 L chains, although this was not formally demonstrated in their work.

It remains an open question whether or not the distribution of Group I and Group II antibodies seen in response to PC-KLH can be attributed to clonotypic differences in affinity for the immunizing antigen, precursor frequency or to additional factors such as idiotype control and intrinsic rate of clonal expansion.

Stage 2: Variations of combinations and somatic mutation. The most prominent feature of the developing antibody response is that the overall population is not static. Systematic studies of both the ARS and OX responses have shown that antibodies seen very early may later be displaced by other clonotypes that were only minor participants in the

early response. The ARS response provides an example where there is more genetic heterogeneity in the initial stages of the response which is soon lost to the dominant presence of a single clonotype that comes to represent 80% of the response (95). The OX response differs from ARS in that the dominant clonotype (V_HOX-1,V_kOX-1) is replaced by antibodies that express new combinations of H and L chains that are the result of reassortment (V_HOX-1 in association with a new V_L and vice versa), as well as entirely new combinations of V genes not observed early in the response (78). This kind of antibody development has been referred to as combinational diffusion (99) and it appears that the anti-p-nitrophenyl(NP) (116) and anti-virus hemagglutinin (HA) (68) responses may follow this pattern. The PC response is clearly dominated by T15 which is the result of a single combination of V_H and V_L genes and it can be seen that the memory response is comprised of new V_H and V_L combinations (14,31,117,118) some of which were found in the primary response but associated with different V_H or V_L genes, and others that represent expression of V genes not detected in the early stages. These findings are presented in detail in Papers 1 and 2 and in the Appendix.

In addition to the shift in population dynamics that is observed during immune maturation, there is extensive somatic mutation of the V regions that participate in the response. A particularly good example of the temporal accumulation of somatic mutations is provided in the anti-OX system where there is a substantial increase in the number of mutations in antibodies obtained during the secondary response compared with those found at earlier times during the primary phase. The increase in somatic mutation paralleled a similar increase in the overall affinity of these late antibodies (72). Such findings are consistent with the notion that

somatic mutations are incurred sequentially and may be selected for by antigen as the response progresses (66).

In keeping with the notion of sequential acquisition of somatic mutations there is some evidence that anti-PC IgG antibodies have more mutations than IgM antibodies (18,32,74) and that there is an increase in affinity for the hapten DPPC in the IgG antibodies that is not seen in the IgMs, the affinity difference is particularly pronounced in M167-like anti-PC antibodies (82). However it is important to note that there was no apparent difference in affinity for the PC hapten alone. It is interesting that the T15-like IgG antibodies contained relatively few V_H mutations (only the NH_2 terminal 36 amino acids of the V_L were sequenced) and did not exhibit a significant increase in affinity for PC or DPPC compared with their germline IgM counterparts (74,82). This may indicate that T15 V genes have been optimally developed during evolution and that the overall structure of these antibodies encoded in the germline is the preferred form of anti-PC antibodies. It is possible that such antibodies do not tolerate significant modifications to their combining sites and that many changes could result in a dimunition of affinity. The studies reported thus far regarding anti-PC antibodies do not allow one to draw conclusions regarding an overall trend in antibody affinity during the antibody response since these were obtained from a late response only. It would be of interest to examine antibodies from the primary and secondary response to assess whether there is a trend toward higher affinity antibodies which parallels an increase in amino acid substitutions.

Stage 3: Clonal selection, part 2. Immune maturation may be best viewed as an evolutionary process that results from continuous selection of the structure(s) of best fit. Although it is not clear what comprises the best structure, it appears that the net result of the somatic processes described, is expansion of the highest affinity clones. It has been suggested that among the new germline combinations found in the late primary response those having the best potential to be modified by somatic mutation into more suitable combining sites will give rise to the very high affinity antibodies that characterize the mature response (78). It has been demonstrated that in the ARS response the Id^{CRI} clonotype comes to dominate late in the response and that somatic variants of this clonotype which are prevalent have equal or greater affinity for ARS than the germline encoded antibody suggesting that affinity selection plays a major role in determining the composition of this response (95).

Group II antibodies appear late in the PC response and despite the original predominance of Group I antibodies can be expanded to be equally represented among anti-PC antibodies in the memory reponse. The genetic heterogeneity that characterizes the binding phenotype of Group II antibodies may allow a greater degree of tolerance against the deleterious effects of somatic mutation; a level of adaptation in which the Group I antibodies (particularly T15) appear to be deficient. Thus as the effects of somatic mutation become widespread there may be a shift in the population resulting in a selective loss of mutated Group I antibodies which may allow Group II antibodies to compete successfully for antigen and to be clonally expanded preferentially.

REFERENCES

1. Pery, P., A. Retit, J. Poulain, and G. Luffau. 1974. Phosphorycholine-bearing components in homogenates of nematodes. Eur. J. Immunol. 4:637-639.

2. Tomasz, A. 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus. Science 157:694-697.

3. Potter, M. 1971. Antigen binding myeloma in mice. Ann. N.Y. Sci. 190:306-321.

4. Claflin, J.L., J. Wolfe, A. Maddalena and S. Hudak. 1984. The murine antibody response to phospholcholine: idiotypes, structures and binding sites. In The Biology of Idiotypes, Plenum Press, New York, p. 171-195.

5. Perlmutter, R.M. 1984. The molecular genetics of phophocholine-binding antibodies. In The Biology of Idiotypes, Plenum Press, New York. p. 59-74.

6. Rudikoff, S. 1983. Immunologlobulin structure - function correlates: antigen binding and idiotypes. Contemp. Topics Molec. Immunol. 9:169-209.

7. Chang, S.P. and M.B. Rittenberg. 1981. Immunologic memory to phosphorycholine in vitro. I. Asymmetric expression of clonal dominance. J. Immunol. 126:975-980.

8. Chang, S.P., M. Brown and M.B. Rittenberg. 1982a. Immunologic memory to phosphorycholine. II. PC-KLH induces two antibody populations that dominate different isotypes. J. Immunol. 128:702-706.

9. Chang, S.P., M. Brown and M.B. Rittenberg. 1982. Immunologic memory to phosphorycholine. III. IgM includes a fine specificity population distinct from TEPC 15. J. Immunol. 129:1559-1562.

10. Claflin, J.L. and S. Rudikoff. 1977. Uniformity in a clonal repertoire: a case for a germline basis of antibody diversity. Cold Spring Harbor Symb. Quant. Biol. 41:725-734.

11. Crews, S., J. Griffin, H. Huang, K. Calame and L. Hood. 1981. A single V_H gene segment encodes the immune response to phosphocholine: somatic mutation is correlated with the class of the antibody. Cell 25:59-66.

12. Cosenza, H. and H. Kohler, 1972. Specific inhibition of plaque formation to phosphorycholine by antibody against antibody. Science 176:1027-1029.

13. Sher, A. and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorycholine. Eur. J. Immunol. 2:319-326.

14. Chang, S.P., R.M. Perlmutter, M. Brown, C.H. Heusser, L. Hood and M.B. Rittenberg. 1984. Immunologic memory to phosphocholine. IV. Hybridomas representative of Group I (T15-like) and Group II (non-T15-like) antibodies utilize distinct V_H genes. J. Immunol. 132:1550-1555.

15. Gearheart, P.J., N.H. Sigal, and N.R. Klinman. 1975. Heterogeneity of the BALB/c antiphosphorycholine antibody repsonse at the precursor cell level. J. Exp. Med. 141:56-71.

16. Claflin, J.L. 1976. Uniformity in the clonal repertoire for the immune response to phosphorycholine in mice. Eur. J. Immunol. 6:669-674.

17. Barstad, P., S. Rudikoff, M. Potter, M. Cohn, W. Konigsberg and L. Hood. 1974. Immunoglobulin structure: Amino terminal sequences of mouse myeloma proteins that bind phosphorycholine. Science 183:962-964.

18. Gearheart, P.J., N.D. Johnson, R. Douglas and L. Hood. 1981. IgG antibodies to phosphorycholine exhibit more diversity than their IgM counterparts. Nature 291:29-34.

19. Leon, M.A. and N.M. Young. 1971. Specificity for phosphorycholine of six murine myeloma proteins reactive with Pneumococcus C polysaccharide and β-lipoprotein. Biochemistry 10:1424-1429.

20. Claflin, J.L., R. Lieberman and J.M. Davie. 1974. Clonal nature of the immune response to phosphorycholine. II. Idiotype specificity and binding characteristics of anti-phosphorycholine antibodies. J. Immunol. 112:1747-1756.

21. Kenny, J.J., G. Guelde, J.L. Claflin and I. Scher. 1981. Altered idiotype response to phosphocholine in mice bearing an X-linked immune defect. J. Immunol. 127:1629-1633.

22. Clough, E.R., D.A. Levy and J.J. Cebra. 1981. CBA/N X BALB/c F_1 male and female mice can be primed to express quantitatively equivalent secondary anti-phosphocholine responses. J. Immunol. 126:387-389.

23. Scher, I. 1982. The CBA/N mouse strain: an experimental model illustrating the influence of the X-chromosome on immunity. Adv. Immunol. 33:1-71.

24. Kishimoto, T., S. Shigemoto, T. Watanabe and Y. Yamamura. 1979. Demonstration of phosphorycholine-specific IgE B cells in CBA/N mice. J. Immunol. 123:1039-1043.

25. Klinman, N.R. and M.R. Stone. 1983. Role of variable region gene expression and environmental selection in determining the anti-phosphorycholine B cell repertoire. J. Exp. Med. 158:1948-1961.

26. Wicker, L.S., G. Guelde, I. Scher and J. Kenny. 1982. Antibodies from the Lyb5 B cell subset predominate in the secondary IgG response to phosphocholine. J. Immunol. 129:950-953.

27. Kenny, J.J., L.J. Yaffe, A. Ahmed and E.S. Metcalf. 1983. Contribution of Lyb-5^+ and Lyb-5^- B cells to the primary and secondary phosphocholine-specific antibody response. J. Immunol. 130:2574-2579.

28. Wicker, L.S., G. Guelde, I. Scher and J.J. Kenny. 1983. The asymmetry in idiotype-isotype expression in the response to phosphocholine is due to divergence in the expressed repertoires of $Lyb5^+$ and $Lyb5^-$ B cells. J. Immunol. 131:2468-2476.

29. Clarke, S.H., J.J. Kenny, D.G. Sieckmann and S. Rudikoff. 1984. Amino acid sequence of a phosphocholine-binding antibody from an immune defective CBA/N mouse employing the T15 V_H region associated with unusual D_H, J_H and V_k segments. J. Immunol. 132:1544-1549.

30. Primi, D. and P.A. Cazenave. 1986. Lack of expression of the V_H S107 gene family in lipopolysaccharide-sensitive B cell subset of X-linked immunodeficiency-defective mice. J. Exp. Med. 164:357-362.

31. Todd, I., S.P. Chang, R.M. Perlmutter, R. Aebersold, C. Heusser, L. Hood and M.B. Rittenberg. 1984. Immunologic memory to phosphocholine V. Hybridomas representative of Group II antibodies utilize V_k 1-3 gene(s). J. Immunol. 132:1556-1560.

32. Perlmutter, R.M., S.T. Crews, R. Douglas, G. Sorenson, N. Johnson, N. Nivera, P.J. Gearheart and L. Hood. 1984. The generation of diversity in phosphorycholine-binding antibodies. Adv. Immunol. 35:1-37.

33. Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature. 302:575-581.

34. Kemp, D.L., B. Tyler, O. Bernard, N. Gough, S. Gerondakis, J.M. Adams and S. Cory. 1981. Organization of genes and spacers within the mouse immunoglobulin V_H locus. J. Molec. Appl. Genet. 1:245-261.

35. Kurosawa, Y. and S. Tonegawa. 1982. Organization, structure and assumbly of immunoglobulin heavy chain diversity DNA segments. J. Exp. Med. 155:201-218.

36. Sakano, H., R. Maki, Y. Kurosawa, W. Roeder and S. Tonegawa. 1980. Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy chain genes. Nature 286:676-683.

37. Cory, S., B.M. Tyler and J.M. Adams. 1981. Sets of immunoglobulin V_k genes homologous to ten cloned V_k sequences: implications for the number of germline V_k genes. J. Mol. Appl. Genet. 1:103-116.

38. Sakano, H., K. Huppi, G. Heinrich and S. Tonegawa. 1979. Sequences at the somatic recombination sites of immunoglobulin light-chain genes. Nature 280:288-294.

39. Reilly, E.B., B. Blomberg, T. Imanishi-Kari, S. Tonegawa and H.N. Eisen. 1984. Restricted association of V and J-C gene segments for mouse λ light chains. Proc. Natl. Acad. Sci. USA 81:2484-2488.

40. Max, E.E., J.G. Seidman and P. Leder. 1979. Sequences of five potential recombination sites encoded close to an immunoglobulin k constant region gene. Proc. Natl. Acad. Sci. USA 76:3450-3454.

41. Early, P., H. Huang, M. Davis, K. Kalame and L. Hood. 1980. An immunoglobulin heavy chain variable region is generated from three segments of DNA: V_H, D and J_H. Cell 19:981-992.

42. Clarke, S.H. and S. Rudikoff. 1984. Evidence for gene conversion among immunoglobulin heavy chain variable region genes. J. Exp. Med. 159:773-782.

43. Huang, H., S. Crews and L. Hood. 1981. An immunoglobulin V_H pseudogene. J. Mol. App. Genet. 1:93-101.

44. Briles, D.E., C. Forman, S. Hudak and J.L. Claflin. 1982. Antiphosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. 156:1177-1185.

45. Riesen, W.F., D.G. Braun and J.C. Jaton. 1976. Human and murine phosphorycholine-binding immunoglobulins: Conserved subgroup and first hypervariable region of heavy chains. Proc. Natl. Acad. Sci. USA 73:2096-2100.

46. Perlmutter, R.M., B. Berson, J.A. Griffin and L. Hood. 1985. Diversity in the germline antibody repertoire. Molecular evolution of the T15 V_H gene family. J. Exp. Med. 162:1998-2016.

47. Cook, W.D., S. Rudikoff, A.M. Giusti and M.D. Scharff. 1982. Somatic mutation in a cultured mouse myeloma cell affects antigen binding. Proc. Natl. Acad. Sci. USA 79:1240-1244.

48. Clarke, S.H., J.L. Claflin and S. Rudikoff. 1982. Polymorphisms in immunoglobulin heavy chains suggesting gene conversion. Proc. Natl. Acad. Sci. USA 79:3280-3284.

49. Clarke, S.H., J.L. Claflin, M. Potter and S. Rudikoff. 1983. Polymorphisms in anti-phosphocholine antibodies reflecting evolution of immunoglobulin families. J. Exp. Med. 157:98-113.

50. Rudikoff, S., M. Pawlita, J. Pumphrey and M. Heller. 1983. Somatic diversification of immunoglobulins. Proc. Natl. Acad. Sci. USA 81:2162-2166.

51. Pollock, B.A., J.A. Kearney, M. Vakil and R.P. Perry. 1984. A biological consequence of variation in the site of D-J_H gene rearrangement. Nature 311:376-379.

52. Berek, C. 1984. The D segment defines the T15 idiotype: the immunoresponse of A/J mice to Pneumococcus pneumoniae. Eur. J. Immunol. 14:1043-1048.

54. Alt, F.W. and D. Baltimore. 1982. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-J_H fusions. Proc. Natl. Acad. Sci. USA 79:4118-4122.

55. Kwan, S.P., S. Rudikoff, J.G. Seidman, P. Leder and M.D. Scharff. 1981. Nucleic acid and protein sequences of phosphocholine-binding eight chains. J. Exp. Med. 153:1366-1369.

56. Rudikoff, S. and M. Potter. 1978. K chain variable region from M167, a phosphorycholine binding myeloma protein. Biochemistry 17:2703-2707.

57. Rudikoff, S., Y. Satow, E. Padlan, D. Davies and M. Potter. 1981. Kappa chain structure from a crystallized murine FAB: Role of joining segment in hapten binding. Mol. Immunol. 18:705-711.

58. Andres, C.M., A. Maddalena, S. Hudak, N.M. Young and J.L. Claflin. 1981. Anti-phosphocholine hybridoma antibodies. II. Functional analysis of binding sites within three antibody families. J. Exp. Med. 154:1584-1598.

59. Kabat, E.A., T.T. Wu, H. Bilofsky, M. Reid-Miller and H. Perry. 1983. Sequences of proteins of immunological interest. U.S. Department of Health and Human Services, National Institute of Health, Bethesda.

60. Satow, Y., G.H. Cohen, E.A. Padlan and D.R. Davies. 1986. Phosphocholine binding immunoglobulin Fab McPc603. An X-ray diffraction study at 2.7Å. J. Mol. Biol. 190:593-604.

61. Gearheart, P.J. and D. Bogenhagen. 1983. Clusters of point mutations are found exclusively around rearranged antibody variable genes. Proc. Natl. Acad. Sci. USA 80:3439-3443.

62. Todd, I., M. Brown and M.B. Rittenberg. 1985. Immunologic memory to phosphorycholine. VI. Heterogeneity in light chain gene expression. Eur. J. Immunol. 15:177-183.

63. Rittenberg, M.B., T.J. Hall and M. Stenzel-Poore. 1986. Immunologic memory to phosphocholine. IX. Utilization of lambda light chains in the memory antibody response to PC-KLH. In preparation.

64. Bothwell, A.L.M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky and D. Baltimore. 1981. Heavy chain variable region contribution to the NP^b family of antibodies: somatic mutation evident in a γ 2a variable region. Cell 24:625-637.

65. Rodwell, J.D. and F. Karush. 1980. Restriction in IgM Expression-I. The V_H regions of equine anti-lactose antibodies. Mol. Immunol. 17:1553-1561.

66. McKean, D., K. Huppi, M. Bell, L. Stoudt, W. Gerhard and M. Weigert. 1984. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinim. Proc. Natl. Acad. Sci. USA 81:3180-3184.

67. Cook, W.D. and M.D. Scharff. 1977. Antigen-binding mutants of mouse myeloma cells. Proc. Natl. Acad. Sci. USA 74:5687-5691.

68. Clarke, S.H., K. Huppi, D. Ruezinsky, L. Stoudt, W. Gerhard and M. Weigert. 1985. Inter- and intraclonal diversity in the antibody response to influenza hemagglutinim. J. Exp. Med. 161:687-704.

69. Pech, M., J. Hochtl, H. Schnell and H. Zachau. 1981. Differences between germline and rearranged immunoglobulin V_k coding sequences suggest a localized mutation mechanism. Nature 291:668-670.

70. Kim, S., M. Davis, E. Sinn, P. Patten and L. Hood. 1981. Antibody diversity: somatic hypermutation of rearranged V_H genes. Cell 27:573-581.

71. Rudikoff, S., M. Pawlita, J. Pumphrey and M. Heller. 1984. Somatic diversification of immunoglobulins. Proc. Natl. Acad. Sci. USA 81:2162-2166.

72. Griffiths, G.M., C. Berek, M. Kaartinen and C. Milstein. 1984. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature 312:271-275.

73. Honjo, T. and S. Habu. 1985. Origin of immune diversity: genetic variation and selection. Ann. Rev. Biochem. 54:803-830.

74. Gearheart, P.J. 1984. The adaptable somatic repertoire. Ann. Immunol. (Inst. Pasteur) 135 C:137-142.

75. Joho, R., H. Gershenfeld and I.L. Weissmann. 1984. Evolution of a multi-gene family of V_k germline genes. Embo. J. 3:185-191.

76. Selsing, E. and U. Storb. 1981. Somatic mutation of immunoglobulin light-chain variable-region genes. Cell 25:47-58.

77. Gershenfeld, H.K., A. Tsukamoto, I.L. Weissman, and R. Joho. 1981. Somatic diversification is required to generate the V_k genes of MOPC 511 and MOPC 167 myeloma proteins. Proc. Natl. Acad. Sci. USA 78:7674-7678.

78. Berek, C., G.M. Griffiths and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. Nature 316:412-418.

79. Simms, J., T.H. Rabbitts, P. Estess, C. Slaughter, P. Tucker and J.D. Capra. 1982. Somatic mutation in genes for the variable portion of the immunoglobulin heavy chain. Science 216:309-311.

80. Near, R.I., E.C. Juszczak, S.Y. Huang, S.A. Sicari, M.N. Margolies and M.L. Gefter. 1984. Expression and rearrangement of homologous immunoglobulin V_H genes in two mouse strains. Proc. Natl. Acad. Sci. USA 81:2167-2171.

81. Caton, A.J., G.G. Brownlee, L.M. Stoudt and W. Gerhard. 1986. Structural and functional implications of a restricted antibody response to a defined antigenic region on the influenza virus hemagglutinin. Embo. J. 5:1577-1587.

82. Rodwell, J.D., P.J. Gearheart and F. Karush. 1983. Restriction in IgM expression. IV. Affinity analysis of monoclonal anti-phosphorylcholine antibodies. J. Immunol. 130:313-316.

83. Rudikoff, A., A.M. Giusti, W.D. Cook and M.D. Scharff. 1982. Single amino aicd substitution altering antigen-binding specificity. Proc. Natl. Acad. Sci. USA 79:1979-1983.

84. Teillaud, J.-L., C. Desaymard, A. Guisti, B. Haseltine, R.R. Pollock, D.E. Yelton, D.J. Zack and M.D. Scharff. 1983. Monoclonal antibodies reveal the structural basis of antibody diversity. Science 222:721-726.

85. Segal, D.M., E.A. Padlan, G.H. Cohen, S. Rudikoff, M. Potter and D.R. Davies. 1974. The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. Proc. Natl. Acad. Sci. USA 71:4298-4302.

86. Diamond, B. and M.D. Scharff. 1984. Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. proc. Natl. Acad. Sci. USA 81:5841-5844.

87. Padlan, E.A., D.R. Davies, S. Rudikoff and M. Potter. 1976. Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. Immunochemistry 13:945-949.

88. Amit, A.G., R.A. Mariuzza, S.E. Phillips and R.J. Poljak. 1986. Three dimensional structure of an antigen antibody complex at 2.8 A resolution. Science 233:747-753.

89. Saul, F., L.M. Amzel and R.J. Poljak. 1978. Preliminary refinement and structural analysis of the Fab fragment from human immunoglobulin New at 2.0Å. J. Biol. Chem. 253:585-597.

90. Marquart, M., J. Deisenhofer, R. Huber and W. Palm. 1980. Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen-binding fragment at 3.0Å and 1.9Å resolution. J. Mol. Biol. 141:369.

91. Padlan, E.A., G.H. Cohen and D.R. Davies. 1985. On the specificity of antibody/antigen interactions: phosphocholine binding to McPC603 and the correlation of three-dimensional structure and sequence data. Ann. Inst. Pasteur Immunol. 136C:271-276.

92. Eisen, H.N. and G.W. Siskind. 1964. Variations in affinities of antibodies during the immune response. Biochemistry 3:996-1007.

93. Eisen, H.N. 1966. The immune response to a simple antigenic determinant. In The Harvey Lectures (Academic Press, New York) 60:1-33.

94. Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495.

95. Wysocki, L., T. Manser and M.L. Gefter. 1986. Somatic evolution of variable region structures during an immune response. Proc. Natl. Acad. Sci. USA 83:1847-1851.

96. Kaartinen, M., G.M. Griffiths, A.F. Markham and C. Milstein. 1983. mRNA sequences define an unusually IgG response to 2-phenyloxazolone and its early diversification. Nature 304:320-324.

97. Pelkonen, J., M. Kaartinen and O. Makela. 1986. Quantitative representation of two germline V genes in the early antibody response to 2-phenyloxazolone. Eur. J. Immunol. 16:106-109.

98. Kaartinen, M., J. Pelkonen and O. Makela. 1986. Several V genes participate in the early phenyloxazolone response in various combinations. Eur. J. Immunol. 16:98-105.

99. Manser, T., L.J. Wysocki, T. Gridley, R.I. Near and M.L. Gefter. 1985. The molecular evolution of the immune response. Immunol. Today 6:95-101.

100. Burnet, M. In The Clonal Selection Theory of Acquired Immunity (Cambridge Univ. Press, London, 1959).

101. Wikler, M., J.-D. Franssen, C. Collignon, O. Leo, B. Mariame, P. Van de Walle, D. DeGroote and J. Urbain. 1979. Idiotypic regulation of the immune system. Common idiotypic specificities between idiotype and antibodies raised against anti-idiotypic antibodies in rabbits. J. Exp. Med. 150:184-195.

102. Jerne, N.K. 1976. The immune system: a web of V domain. In The Harvey Lectures (Academic Press, New York) 70:93.

103. Manser, T., S.-H. Huang and M.L. Gefter. 1984. Influence of clonal selection on the expression of immunoglobulin variable region genes. Science 226:1283-1288.

104. Owen, J., N.H. Sigal and N.R. Klinman. 1982. Heterogeneity of the BALB/c IgM anti-phosphorylcholine antibody response. Nature 295:347-348.

105. Sigal, N.H., P.J. Gearheart and N.R. Klinman. 1975. The frequency of phosphorylcholine-specific B cells in conventional and germfree BALB/c mice. J. Immunol. 114:1354-1358.

106. Sigal, N.H., A.R. Pickard, E.S. Metcalf, P.J. Gearheart and N.R. Klinman. 1977. Expression of phosphorycholine specific B cells during murine development. J. Exp. Med. 146:933-948.

107. Klinman, N.R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. J. Exp. Med. 136:241-260.

108. Anderson, J., O. Sjoberg and G. Moller. 1972. Induction of immunoglobulin in vitro by lipopolysaccharides. Eur. J. Immunol. 2:349-353.

109. Primi, D., A.M. Drapier and P.A. Cazenave. 1986. Differential expression of sets of highly homologous variable region gene products in selected and pre-immune repertoires of inbred mouse strains. J. Exp. Med. 163:1051-1063.

110. Levy, A. 1984. Studies on antibody repertoire: ontogenic development of mitogen-reactive B cells producing phosphorycholine-specific and/or T15-associated 10/13 idiotype positive antibodies. Eur. J. Immunol. 14:1141-1144.

111. Levy, M. 1984. Frequencies of phosphorylcholine-specific and T15-associated 10/13 idiotype-positive B cells within lipopolysaccharide-reactive B cells of adult BALB/c mice. Eur. J. Immunol. 14:864-868.

112. Manser, T. and M.L. Gefter. 1984. Isolation of hybridomas expressing a specific heavy chain variable region gene segment by using a screening technique that detects mRNA sequences in whole cell lysates. Proc. Natl. Acad. Sci. USA 81:2470-2474.

113. Fung, J. and H. Kohler. 1980. Immune response to phosphorylcholine. VII. Functional evidence for three separate B cell subpopulations responding to TI and TD PC-antigens. J. Immunol. 125:640-646.

114. Ettinger, H.M. and C.H. Heusser. 1986. T15 dominance in BALB/c mice is not controlled by environmental factors. J. Immunol. 136:1988-1991.

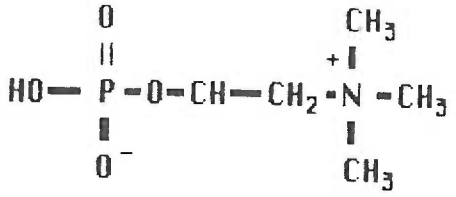
115. Ettinger, H.M., M.H. Julius and C.H. Heusser. 1982. Mechanism of clonal dominance in the murine antiphosphorylcholine response. J. Immunol. 128:1685-1691.

116. Boersch-Supan, M.E., S. Agarwal, M.E. White-Scharf and T. Imanishi-Kari. 1985. Heavy chain variable region. Multiple gene segments encode anti-4-(hydroxy-3-nitrophenyl)acetyl idiotypic antibodies. J. Exp. Med. 161:1272-1292.

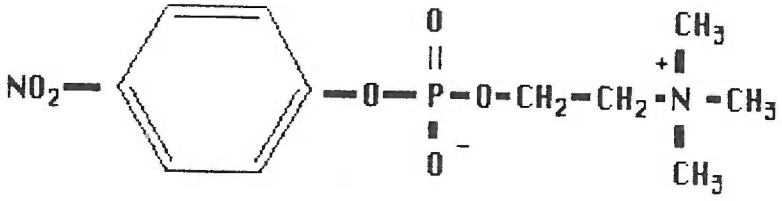
117. Brown, M., M. Stenzel-Poore and M.B. Rittenberg. 1985. Immunologic memory to phosphocholine. VII. Lack of T15 V1 gene utilization in Xid anti-PC hybridomas. J. Immunol. 135:3558-3563.

118. Rittenberg, M.B., R.W. Glanville, R.H. Aebersold, J.P. Chang and M. Brown. 1986. Immunologic memory to phosphorylcholine (PC). VIII. Expression of the V_H-12 gene product in the response to PC-keyhole limpet hemocyanin. Eur. J. Immunol. 16:503-507.

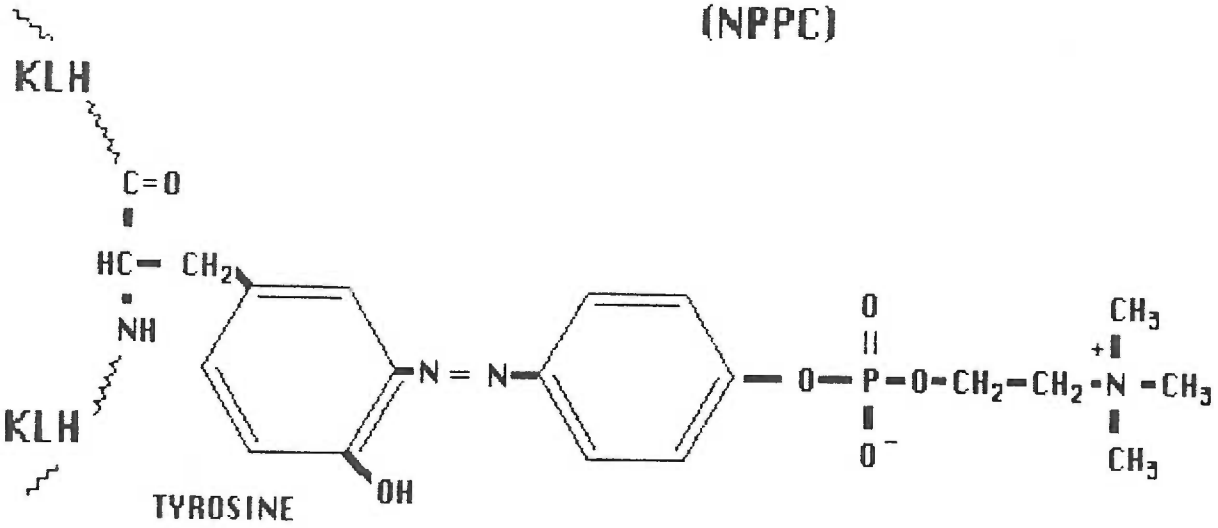
Literature Review Figure 1. Structures of hapten analogs and the phosphocholine-protein (PC-protein) immunizing antigen. Phosphocholine (PC) and the nitrophenyl derivative p-nitrophenyl phosphocholine (NPPC) were used to inhibit binding of anti-PC antibodies to PC-protein. The immunizing form of PC is shown coupled to the protein carrier keyhole limpet hemocyanin (KLH) via a diazotized tyrosine bridge.



PHOSPHOCHOLINE
(PC)



p-NITROPHENYL PHOSPHOCHOLINE
(NPPC)



PHOSPHOCHOLINE-PROTEIN CONJUGATE
(PC-KLH)

Paper 1

Immunologic Memory to Phosphocholine. VII. Lack of T15 V1 Gene Utilization in Xid Anti-PC Hybridomas

ABSTRACT

CBA/N mice carrying the Xid defect fail to make antibodies expressing the T15 idiotype in response to immunization with PC-KLH. Antibodies predominating in the Xid response have binding properties characteristic of Group II antibodies that emerge in the memory response in BALB/c; the prototype Group II antibody utilizes a V_H gene product distinct from the V1 gene product expressed by T15 idiotype positive antibodies. In order to examine V_H gene usage in the anti-PC response of Xid B cells, hybridomas were produced from Xid mice immune to PC-KLH. Four hybridomas possessing properties typical of the predominant Group II antibody response in Xid mice and two representing minor components of the response were studied. Analysis of DNA by Southern blot hybridization revealed that none of the hybridomas utilized the T15 V1 gene segment, nor did they share use of a common VDJ gene product. These results indicate that Xid Group II antibodies either make use of different V_H gene segments or use the same V_H in combination with various D and J_H segments.

INTRODUCTION

The immune memory response to PC-KLH in BALB/c mice is dominated by two major groups of antibodies distinguishable by binding characteristics and idiotype (1,2). Group I antibodies have high affinity for the free hapten PC, and are associated preferentially with the IgM, IgG3, and IgA isotypes. Myelomas and hybridomas representative of Group I employ the T15 heavy chain variable region sequence (encoded by the V1 gene segment) in combination with V_K8, V_K22 or V_K24 light chains (3,4);only those which utilize V_K22 light chains are T15 idiotype positive. Group II antibodies are negative for the T15 idiotype, bind nitrophenyl phosphocholine (NPPC) with higher affinity than PC, and are predominantly IgG1 and IgG2. The Group II hybridoma antibodies we have examined so far appear to utilize V_H genes different from the T15 V1 gene (5), while light chain usage involves gene products found in Group I in addition to V_k1-3 and $\lambda2$ (6).

Because CBA/N mice produce little $T15^+$ antibody, mice carrying the Xid defect have been used as a tool for examining mechanisms controlling T15 gene expression in the PC system. In contrast to their female counterparts, defective F1 male Xid mice produce $T15^-$ antibodies that are predominantly of the IgG isotype in both the primary and secondary response to PC-KLH while failing to make normal levels of IgM or IgG3 antibodies (7,8). This altered idiotype and isotype response to PC results in production of antibodies in the memory response to PC-KLH that are mainly of the IgG1 and IgG2 subclasses and Group II in phenotype (9,10). A substantial amount of antibody is also produced which has low affinity for both PC and NPPC and has been called Group III (5,10).

Although negative for the T15 idiotype, Xid B cells can produce antibody possessing heavy chain V_H determinants common to members of the T15 family (7,10). This is consistent with a finding that the V1 allele present in CBA/N mice was functionally rearranged in a hybridoma with low affinity for PC (11). The expressed V_H gene utilized D and J segments not usually associated with V1 in combination with a V_K3 light chain. We have previously described Group II hybridomas from F1 Xid mice that employed light chains comparable to those used by Group I antibodies (6). The question can be asked whether these hybridomas are T15 negative because they have not functionally rearranged the V1 gene segment, or whether they utilize the V1 gene in conjunction with unique D or J_H segments that render them T15 idiotype negative. In this paper we examine hybridomas from CBA/N x BALB/c F1 male mice, most of which typify the memory response in binding specificity, idiotype, and isotype (i.e. Group II, $T15^-$, IgG) and some of which represent minor components of the Xid response (i.e. IgM Group I and IgG3 Group II). These hybridomas were analyzed by Southern blot analysis using a probe specific for the T15 V1 gene. In all cases the T15 V_H gene remained germline in configuration and thus could not be employed for antibody production.

MATERIALS AND METHODS

Hybridoma and myeloma proteins. (CBA/N x BALB/c)F1 male mice bred in our animal facility were immunized for induction of memory responses to PC-KLH (2). Four days before fusion mice were challenged with an i.v. injection of 100 μg PC-KLH in saline. The production, selection, and cloning of hybridomas from mice immune to PC-KLH by fusion of spleen

cells with the SP2/0-Ag14 fusion partner (SP2) has been described previously (5,12). The hybridomas and TEPC 15, MOPC 511, and McPC 603 myelomas were grown in mice as ascites. Antibodies were purified by affinity chromatography on PC-Sepharose 4B columns as described (2).

Determination of isotype (1), idiotype (2), binding to the pneumococcus R36A (5) and hapten inhibition profiles (1) of the hybridoma proteins by ELISA were determined as described previously. TC54 cells (13) were provided by Drs. C. Desaymard and M.D. Scharff; F6.3 (14) ascites fluid was provided by Dr. D. Levitt; AB1-2 cells (15) were from the American Type Culture Collection, Rockville, MD. Anti-idiotype reagents were purified on protein A-Sepharose 4B columns (16).

DNA blots. For isolation of DNA, TEPC 15, SP2, and PCG1-1 (5), cells were grown as subcutaneous tumors in BALB/c mice. The Xid hybridoma cells were grown in tissue culture in RPMI 1640 + 15% fetal calf serum. DNA was extracted by the method of Blin and Stafford (17). The isolated DNA's were digested with Hind III or EcoR1 in 10 units enzyme/μg DNA for 4-6 hr at 37°C, electrophoresed in 0.8% agarose gels, and blotted (18) to nitrocellulose (Bio-Rad, Richmond, CA). Prehybridizations (16 hr) and hybridizations (24 hr) were performed at 42°C in 6X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 5X Denhardt's solution (1X = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 50% formamide, 10% dextran sulfate, 0.01 M EDTA, and 100 μg/ml denatured salmon sperm DNA. Filters were first washed in 2X SSC, 0.1% SDS at room temperature and then in 0.2X SSC, 0.1% SDS at either 50°C (J_H probe), or 65°C (V1 probe). A 3.2 kb probe containing all four BALB/c J_H genes cloned into pBR322 (19) was provided by Drs. R. Perlmutter and L. Hood. A 0.6 kb probe containing 5'

flanking sequences of the V1 gene cloned into M13mp8 was provided by G. Siu and Dr. L. Hood. Probes were labeled with ^{32}P-dATP to a specific activity of 2-5 x 10^8 cpm/μg and used at 1.5 x 10^6 cpm/ml in the hybridization mixture.

RESULTS

Binding specificity of Xid hybridomas. Fusion of SP2/0 cells with spleen cells from (CBA/N x BALB/c)F1 male mice immune to PC-KLH resulted in hybridomas secreting anti-PC antibodies of every isotype except IgA and IgE. Antibodies of each isotype were chosen for further study. Proteins purified on PC-Sepharose were analyzed for their binding properties by inhibition of binding to PC-histone coated plates by varying concentrations of PC or NPPC in an ELISA (Fig. 1). Four of the hybridomas studied have binding profiles representing antibodies produced in high proportions by Xid mice in response to immunization with PC-protein (10). PCG2a-1, PCG2b-2, and PCG1-3 all have much higher affinity for NPPC than PC and are thus classified as Group II (1). One of the γ2b hybridomas, PCG2b-1 could not be inhibited by free PC or NPPC, but could be inhibited completely by PC-BSA (not shown); this type of antibody represents a large proportion of IgM and a smaller proportion of IgG antibodies produced by Xid immune spleen cells. Surprisingly a Group II hybridoma of the γ3 isotype was produced, unusual because of the rarity of this isotype in the Xid mouse. Although the binding of this hybridoma PCG3-2 is representative of Group II antibodies, it binds NPPC with higher affinity than any of the other Group II monoclonal antibodies. The IgM hybridoma PCM24 is also unusual in that it possesses

hapten specificity typical of Group I antibodies (i.e. NPPC/PC = 9.5) and thus represents a kind of antibody found only in low amounts in the Xid response to PC-KLH (10).

Idiotype expression of Xid hybridomas. The purified hybridoma proteins were tested for expression of the T15 idiotype by an ELISA competition assay using monoclonal anti-T15 reagents. AB1-2 and F6.3 are specific for determinants requiring the combination of T15 heavy and light chains (14,15); TC54 reacts with a common V_H determinant present on TEPC 15, MOPC 167, MOPC 511, and perhaps McPC 603 protein although this latter protein reacts weakly at best (5,13). None of the Xid hybridoma proteins could compete with T15 for binding to any of the three anti-idiotype antibodies (Fig. 2). This was an expected result for the reagents recognizing private T15 determinants since none of these hybridomas use the T15 light chain, V_K22 (6). The lack of expression of T15 V_H determinants as indicated by the failure of the hybridoma proteins to react with the V_H specific monoclonal TC54 could result from the expression of V_H gene segments other than V1 or, alternatively, by the use of a V1 gene which has undergone somatic mutation or combined with D or J_H segments that render the antibodies idiotype negative. These possibilities were distinguished by analyzing hybridoma DNA for rearrangement of the V1 gene.

Germline configuration of the V1 gene in Xid hybridomas. Analysis of EcoR1 cut DNA from two PC-binding myelomas (M603 and M167) whose V_H genes are derived from the germline T15 V_H gene segment showed that the rearranged V1 genes can be distinguished from germline by Southern blot analysis (5,20). However, because of the closeness in size of the germline (7.8 kb) and rearranged (7.2 kb) fragments in EcoR1 digested

DNA, we chose instead to use the restriction enzyme Hind III to determine rearrangement of the V1 gene in DNA from the Xid hybridomas. Southern blots of Hind III digested genomic DNA were hybridized to a 5' flanking region probe specific for the V1 gene (Fig. 3a,b). In contrast to the myeloma T15 which has a 5.0 kb rearranged band, the hybridomas PCM24, PCG2b-1, PCG2a-1, PCG1-3, PCG2b-2, and PCG3-2 all contain the V1 gene in germline configuration. Because the hybridomas were derived from F1 mice there are two possible restriction fragments representing the germline arrangement of the V1 gene, which correspond to the germline fragments from the parental CBA/N (6.6 kb) or BALB/c (7.1 kb) liver. Thus there is a Hind III restriction polymorphism that distinguishes the V1 gene in these two strains. It cannot be determined from this analysis if the BALB/c germline V1 gene observed in the hybridomas is contributed by the Xid spleen cell or by the SP2 fusion partner which is of BALB/c origin. It is evident, however, that the CBA/N germline V1 gene (6.6 kb band) has been lost from two of the hybridomas, PCM24 and PCG2a-1, either by chromosome loss during establishment of the hybridomas or by deletion of the V1 gene during rearrangement of a gene located 5' to V1.

V_H rearrangements in Xid hybridomas. To predict if the hybridoma antibodies are derived from rearrangement of the same or different V_H gene segments, Southern blots of EcoR1 cut DNA were hybridized to a probe containing all four J_H germline segments (Fig.4). This probe hybridizes to a single 6.3 kb fragment containing the germline J_H sequences because EcoR1 does not cut within the J_H locus (21) and to a 5.7 kb fragment containing a rearranged J_H fragment from SP2. Restriction fragments containing identical rearranged V-J_H gene segments will comigrate while fragments containing the same V gene segment rearranged to different J_H

segments will differ by the distance between the various J_H gene segments. Figure 4 shows the results of this analysis using DNA from the six Xid hybridomas, the T15 myeloma, and PCG1-1, a BALB/c hybridoma with binding properties similar to the Group II Xid hybridomas (5). None of the Xid hybridomas have retained a J_H gene segment in germline configuration, and all of them have the rearranged J_H contributed by the SP2 fusion partner. Although it cannot be determined from this analysis which bands contain the productively rearranged V_H gene, it is clear that no single V_H gene rearrangement is common to all the hybridomas. Several hybridomas do, however, contain rearranged bands of identical size. Most notable are the 4.5 kb band present in PCG1-1, PCG2b-1, and PCG2b-2 and the 3.0 kb band present in PCG2a-1 and PCG3-2. The hybridoma PCG2b-1 also has a restriction fragment co-migrating with the 7.2 kb productively rearranged band (20) from the T15 myeloma; however, as shown above (Fig. 3), PCG2b-1 does not utilize the V1 gene. Either these Group II Xid hybridomas use V_H genes distinct from one another or they use the same V_H gene rearranged to different J_H segments. These alternatives cannot be resolved by the present analysis. The only rearranged band in PCG3-2 (3.0 kb) is close enough in size to the 4.5 kb rearranged band present in PCG1-1, PCG2b-1 and PCG2b-2 as to be accounted for by a J_H rearrangement since the difference between EcoR1 fragments produced by a shared V_H gene rearranged to J_H1 or J_H4 is approximately 1.4 kb (22).

Properties of the Xid hybridomas. A summary of the properties of the six Xid hybridoma antibodies characterized in this study is presented in Table I. The NPPC/PC ratios determined from the binding curves presented in Figure 1 as well as the lack of binding to pneumococcus R36A are given. Data for the T15 family of proteins representing the Group I

specificity are included for comparison of binding properties as well as data for the expression of public and private idiotype determinants. The results indicating light chain utilization are taken from a previous analysis by isoelectric focusing (6).

DISCUSSION

The Xid defect is manifested by the production of low amounts of IgM and IgG3, and by the lack of dominance of the T15 idiotype in antibodies produced in response to PC-KLH. A priori, there is no apparent reason at a molecular level for failure to produce the T15 type of antibody. The CBA/J and CBA/N strains both contain alleles of the T15 gene family in the germline detectable by hybridization with an S107 V_H probe that hybridizes strongly to the four members of the T15 gene family present in BALB/c liver (11). Moreover, it has been shown that cells capable of giving rise to T15 anti-PC antibodies are present in normal proportions among surface Ig^- bone marrow cells of Xid mice based on adoptive transfer of such cells prior to exposure to antigen (23). Thus although not analyzed at a molecular level it appears that the Xid mouse can rearrange the V1 gene at a normal frequency and that the heavy chain generated can combine with a V_K22 light chain to produce a functional $T15^+$ antibody. This would be consistent with the finding of low numbers of $T15^+$ PFC in Xid mice (10), by the isolation of some as yet uncharacterized $T15^+$ hybridomas (11), and by induction of T15+ IgE with low doses of PC-KLH (8,24).

It has been suggested that the non-T15 clones expanded in the CBA/N mouse by PC antigens result from use of the V1 allele rearranged to

unusual D or J segments or to the use of non-T15 light chains (11). The detection of T15 V_H^+, T15 idiotype negative antibodies in serum and spleen of CBA/N mice (7,10) implies that the allele of V1 present in the CBA/N mouse (11) can productively rearrange to produce a functional non-T15-like antibody. This has been demonstrated by isolation of a hybridoma with low affinity for PC (11). On the other hand, PC binding antibodies with V_H gene segments other than V1 have been described (5,25,26) although one of these (25) utilized V11 which is a member of the T15 V_H gene family. Thus it was of interest to characterize the T15$^-$ hybridomas produced from Xid mice immune to PC-KLH. These hybridoma antibodies could be T15$^-$ by 1) use of V_H gene segments other than V1, 2) rearrangement of V1 to D or J_H segments other than those used by T15, or 3) use of T15 V_H in combination with a light chain other than V_K22. Our previous analysis by isoelectric focusing of the light chain usage in Xid hybridomas indicated that V_K22 was not utilized by such hybridomas produced from a memory response to PC-KLH; they did however use light chains related to those employed by the T15 family of antibodies, i.e. V_K24 and V_K8, in addition to V_K1-3 and λ2 which are distinct from the T15 family of light chains (6). Analysis of the heavy chains of these antibodies by hybridization of a 5' flanking region probe for the V1 gene segment to Southern blots of Hind III-cut hybridoma genomic DNA indicates that the V1 gene remains in germline configuration in all of the Xid hybridomas. Thus these antibodies are formed by productive rearrangement of a gene or genes other than V1, resulting in antibodies differing from T15 both in idiotype and binding specificity. However, the similarity in binding properties detected by hapten inhibition and lack of binding to

R36A does not necessarily reflect use of a V_H gene product common to all these hybridomas.

One of the Group II Xid hybridomas PCG3-2, an IgG3, is characteristic of the Xid response in binding properties but is of an isotype normally underrepresented among Xid antibodies (27). This hybridoma uses a V_H gene segment distinct from T15 but has a J_H rearrangement in common with PCG2a-1 (Fig. 4). The predicted use of common V_H genes by these two antibodies is interesting in view of their distinct affinity differences and implies an important role for the light chain and/or somatic mutation affecting the combining site of these antibodies. PCG2a-1 which expresses a V_K1-3 light chain binds NPPC poorly, while PCG3-2 which utilizes a λ2 light chain has the highest affinity for NPPC of any of our Group II hybridomas (6).

The IgM monoclonal antibody studied, PCM24, possesses binding characteristics for PC analogs typical of Group I, but unlike the Group I BALB/c hybridomas and myelomas (5) does not bind R36A. Although PCM24 does not have the V1 gene segment rearranged, it has binding properties in common with a hybridoma of CBA/J origin that utilizes an allele of V11, a member of the T15 gene family (26). Use of this gene would be consistent with the loss of the germline CBA/N V1 gene in PCM24 since the other members of the T15 V_H gene family lie 5' the V1 gene (28), and would thus delete V1 during rearrangement to the D segment. Preliminary data (unpublished) using a probe for the T15 coding region indicate that PCM24 has rearranged a member of the T15 V_H gene family other than V1.

We have shown previously that the Group II antibodies produced in BALB/c and (CBA/N x BALB/c) F1 male mice are more heterogeneous in their light chain usage than Group I, utilizing V_K1-3, λ, V_K8, and V_K24-related

light chains (6). Analysis of rearranged V_H genes in the Xid hybridomas indicates that some of the binding site diversity seen within the Group II hybridomas results from use of more than one V_H gene distinct from T15 V1 or from use of a common V_H gene rearranged to different J_H segments. Previous studies of BALB/c hybridomas indicated that antibodies of Group II specificity can be derived from at least two different V_H genes, one group being somatic variants of V1 (29) and the other employing genes distinct from the T15 family (5). Experiments by Owen et al. (30) indicate that the $T15^-$ IgM anti-PC antibodies present in BALB/c mice may represent over 100 different clonotypes. These could be generated by somatic mutation of germline IgM sequences or by use of additional gene segments. Our results show that V_H gene usage by $T15^-$ Xid hybridomas does not include the V1 gene segment nor are the Group II binding specificities exhibited by these hybridomas derived from use of a particular VDJ combination. Thus studies to define the relative contribution of various V_H gene segments will aid in understanding the molecular basis of diversity in the memory response to PC antigens.

Acknowledgements: We thank L. Jobe and Dr. U. Bruderer for critical review of the manuscript. We are also grateful to Dr. C. Faust for his invaluable instruction and advice in molecular biology techniques. We also thank Drs. C. Chang, R. Perlmutter, L. Hood, and G. Siu for providing probes and advice on their use.

REFERENCES

1. Chang, S.P., M. Brown, and M.B. Rittenberg. 1982. Immunologic memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. J. Immunol. 128:702.
2. Chang, S.P., M. Brown, and M.B. Rittenberg. 1982. Immunologic memory to phosphorylcholine. III. IgM includes a fine specificity population distinct from TEPC 15. J. Immunol. 129:1559.
3. Crews, S., J. Griffin, H. Huang, K. Calame, and L. Hood. 1981. A single V_H gene segment encodes the immune response to phosphorylcholine: Somatic mutation is correlated with the class of the antibody. Cell 25:59.
4. Gearhart, P.J., N.D. Johnson, R. Douglas, and L. Hood. 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. Nature 291:29.
5. Chang, S.P., R.M. Perlmutter, M. Brown, C.H. Heusser, L. Hood, and M. B. Rittenberg. 1984. Immunologic memory to phosphocholine. IV. Hybridomas representative of Group I (T15-like) and Group II (non-T15-like) antibodies utilize distinct V_H genes. J. Immunol. 132:1550.
6. Todd, I., M. Brown, and M. B. Rittenberg. 1985. Immunologic memory to phosphocholine. VI. Heterogeneity in light chain gene expression. Eur. J. Immunol. 15:177.
7. Kenny, J.J., L.J. Yaffe, A. Ahmed, and E.S. Metcalf. 1983. Contribution of Lyb 5^+ and Lyb 5^- B cells to the primary and secondary phosphocholine-specific antibody response. J. Immunol. 130:2574.

8. Clough, E.R., D.A. Levy, and J.J. Cebra. 1981. CBA/N x BALB/cJ F1 male and female mice can be primed to express quantitatively equivalent secondary anti-phosphocholine responses. J. Immunol. 126:387.

9. Wicker, L.S., G. Guelde, I. Scher, and J.J. Kenny. 1982. Antibodies from the Lyb-5^- B cell subset predominate in the secondary IgG response to phosphocholine. J. Immunol. 129:950.

10. Wicker, L.S., G. Guelde, I. Scher, and J.J. Kenny. 1983. The asymmetry in idiotype-isotype expression in the response to phosphocholine is due to divergence in the expressed repertoires of Lyb-5^+ and Lyb-5^-B cells. J. Immunol. 131:2468.

11. Clarke, S.H., J.J. Kenny, D.G. Sieckmann, and S. Rudikoff. 1984. Amino acid sequence of a phosphocholine-binding antibody from an immune defective CBA/N mouse employing the T15 V_H region associated with unusual D_H, J_H, and V_H segments. J. Immunol. 132:1544.

12. Bell, E.B., M. Brown, and M.B. Rittenberg. 1983. In vitro antibody synthesis in 20 µl hanging drops: Initiation of secondary responses and a simple method of cloning hybridomas. J. Immunol. Meth. 62:137.

13. Desaymard, C., A.M. Giusti, and M.D. Scharff. 1984. Rat anti-T15 monoclonal antibodies with specificity for V_H- and V_H-V_L epitopes. Mol. Immunol. 21:961.

14. Wittner, M.K., M.A. Bach, and H. Kohler. 1982. Immune response to phosphorylcholine. IX. Characterization of hybridoma anti-TEPC 15 antibodies. J. Immunol. 128:595.

15. Kearney, J.F., R. Barletta, Z.S. Quan, and J. Quintans. 1981. Monoclonal vs. heterogeneous anti-H-8 antibodies in the analysis of

the anti-phosphocholine response in BALB/c mice. Eur. J. Immunol. 11:877.

16. Ey, P.L., S.J. Prowse, and C.R. Jenkin. 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 15:429.

17. Blin, N., and D.W. Stafford. 1976. A general method for the isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. 3:2303.

18. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503.

19. Calame, K., J. Rogers, P. Early, M. Davis, D. Livant, R. Wall and L. Hood. 1980. Mouse Cμ heavy chain immunoglobulin gene segment contains three intervening sequences separating domains. Nature 284:452.

20. Kim, S., M. Davis, E. Sinn, P. Patten, and L. Hood. 1981. Antibody Diversity: Somatic hypermutation of rearranged V_H genes. Cell 27:573.

21. Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. 1980. An immunoglobulin heavy chain variable region is generated from three segments of DNA: V_H,D and J_H. Cell 19:981.

22. Sakano, H., R. Maki, Y. Kurosawa, W. Roeder, and S. Tonegawa. 1980. Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy chain genes. Nature 286:676.

23. Klinman, N.R. and M.R. Stone. Role of variable region gene expression and environmental selection in determining the anti-phosphorylcholine B cell repertoire. J. Exp. Med. 158:1948.

24. Kishimoto, T., S. Shigemoto, T. Watanabe, and Y. Yamamura. 1979. Demonstration of phosphorylcholine-specific IgE B cells in CBA/N mice. J. Immunol. 123:1039.

25. Kocher, H.P., C. Berek, M.H. Schreier, H. Cosenza, and J-C. Jaton. 1980. Phosphorylcholine-binding hybridoma proteins of normal and idiotypically suppressed BALB/c mice II. Variable region N-terminal amino acid sequences. Eur. J. Immunol. 10:264.

26. Clarke, S.H., J.L. Claflin, and S. Rudikoff. 1982. Polymorphisms in immunoglobulin heavy chains suggesting gene conversion. Proc. Natl. Acad. Sci. 79:3280.

27. Perlmutter, R.M., M. Nahm, K.E. Stein, J. Slack, I. Zitron, W.E. Paul and J.M. Davie. 1979. Immunoglobulin subclass-specific immunodeficiency in mice with an X-linked B-lymphocyte defect. J. Exp. Med. 149:993.

28. Perlmutter, R.M., S.T. Crews, R. Douglas, G. Sorensen, N. Johnson, N. Nivera, P.J. Gearhart, and L. Hood. 1984. The generation of diversity in phosphorylcholine-binding antibodies. Advances in Immunology 35:1.

29. Rodwell, J.D., P.J. Gearhart, and F. Karush. 1983. Restriction in IgM expression IV. Affinity analysis of monoclonal anti-phosphoryl-choline antibodies. J. Immunol. 130:313.

30. Owen, J.A., N.H. Sigal, and N. Klinman. 1982. Heterogeneity of the BALB/c IgM anti-phosphorylcholine antibody response. Nature 295:347.

Figure 1. Hapten inhibition profiles of Xid hybridoma anti-PC antibodies. Binding of purified hybridoma antibodies to PC-protein was measured in an ELISA in the presence of varying concentrations of the haptens PC (•-•) and NPPC (o--o).

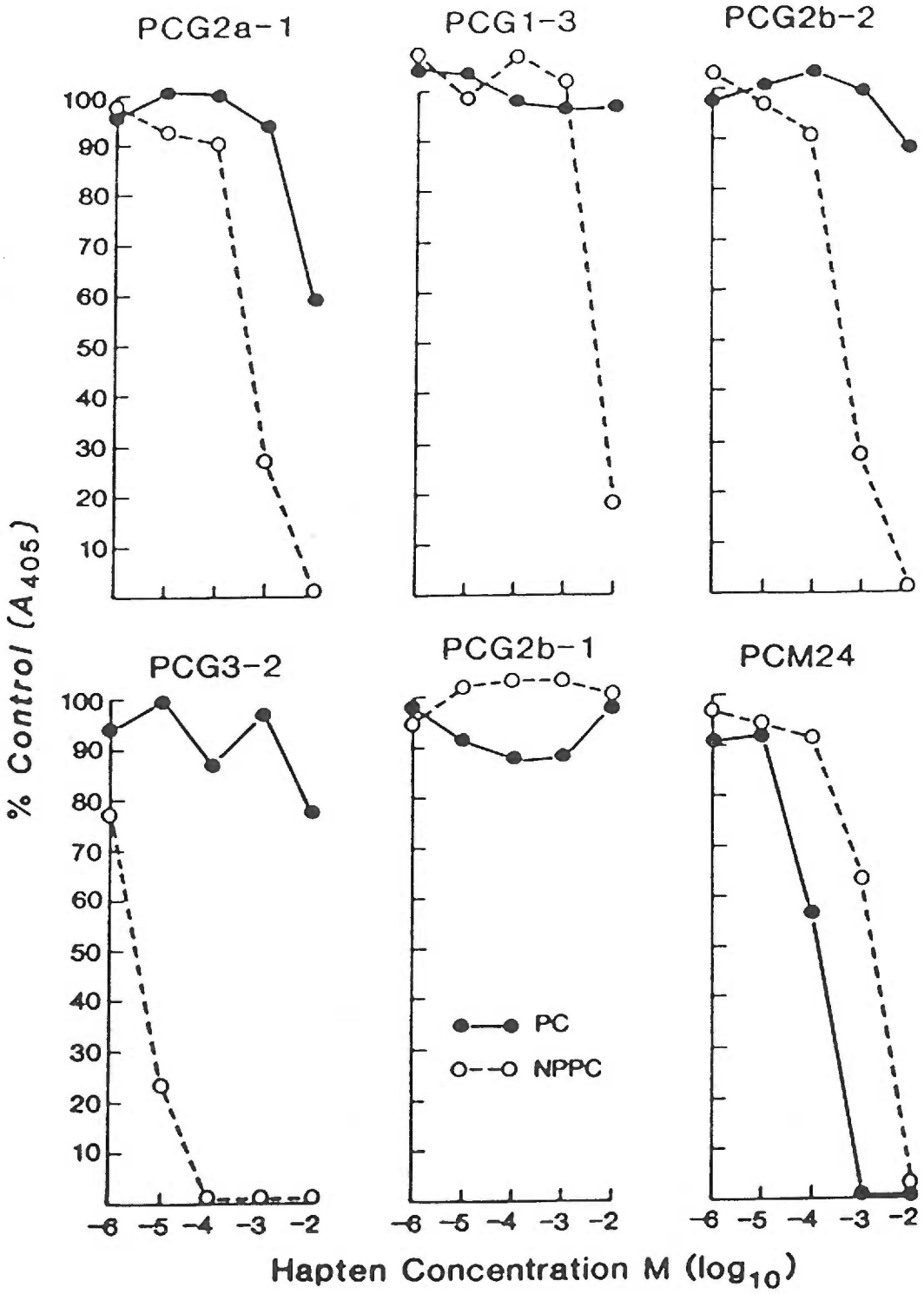

PCG2a-1
PCG1-3
PCG2b-2
PCG3-2
PCG2b-1
PCM24
% Control (A_{405})
100
90
80
70
60
50
40
30
20
10
-6
-5
-4
-3
-2
PC
NPPC
Hapten Concentration M ($\log_{10}$)

Figure 2. Lack of expression of T15 idiotopes by Xid hybridoma anti-PC antibodies. Ability of alkaline phosphatase conjugated TEPC15 protein to bind to anti-idiotype coated wells in the presence of purified unlabeled hybridoma protein or T15 as inhibitor was determined in an ELISA. TEPC15 (●-●); PCG2b-2 (o--o); PCG1-3 (■-■); PCM24 (□--□);PCG3-2 (▲-▲); PCG2a-1 ($^{\triangle}$--$^{\triangle}$); PCG2b-1 (▼-▼).

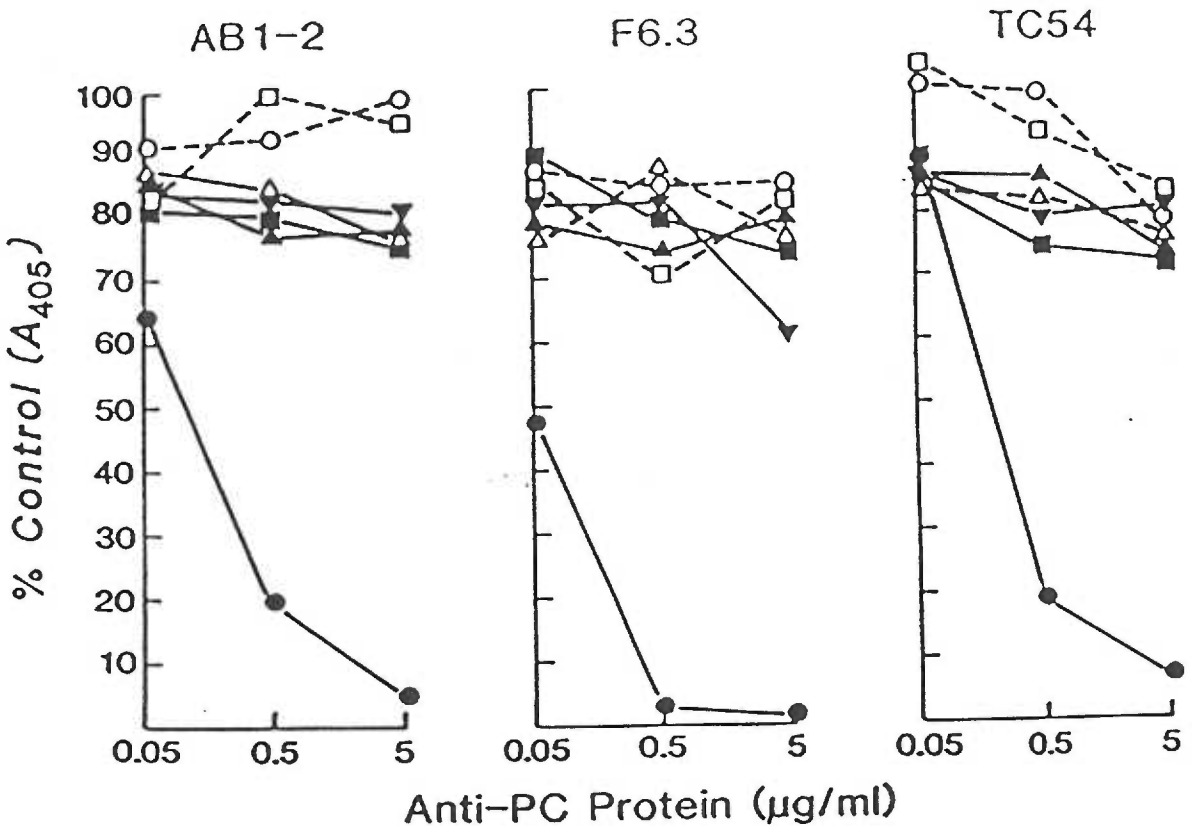
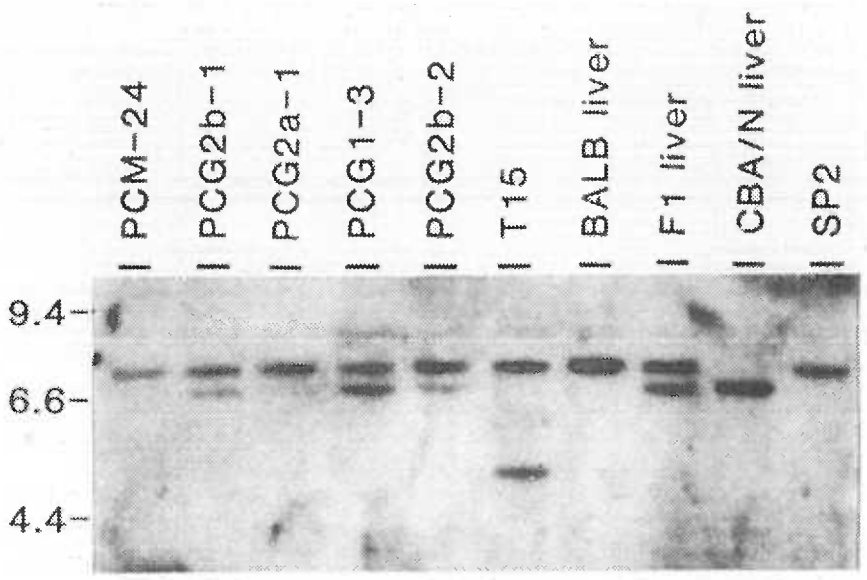

AB1-2
F6.3
TC54
100
90
80
70
60
50
40
30
20
10
% Control (A_{405})
0.05
0.5
5
Anti-PC Protein (μg/ml)

Figure 3a,b. Xid hybridomas do not contain a rearranged V1 gene segment. Southern blots of 2 μg of Hind III-digested DNA were hybridized to a probe containing 5' flanking sequences of the V1 gene. Sources of DNA are indicated above each lane. The positions of DNA size markers (kb) are indicated at the left of each figure.

A


PCM-24
PCG2b-1
PCG2a-1
PCG1-3
PCG2b-2
T15
BALB liver
F1 liver
CBA/N liver
SP2
9.4
6.6
4.4

B

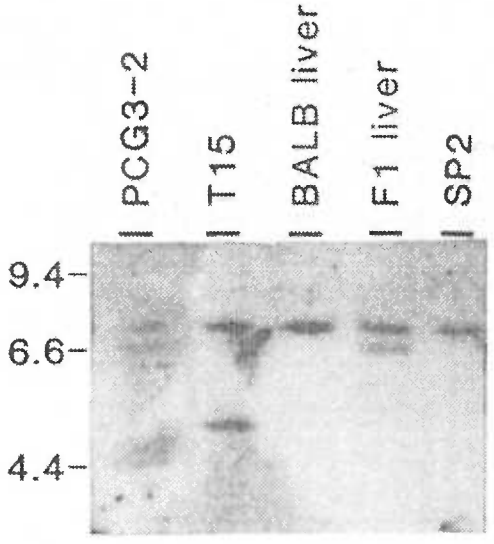

PCG3-2
T15
BALB liver
F1 liver
SP2
9.4
6.6
4.4

Figure 4. J_H rearrangement in the Xid hybridomas. Southern Blots of EcoR1 digested DNA (7 μg/lane) were hybridized with a probe containing all four J_H genes. Sources of DNA are indicated above each lane. The positions of DNA size markers (kb) are indicated at the left of the figure.

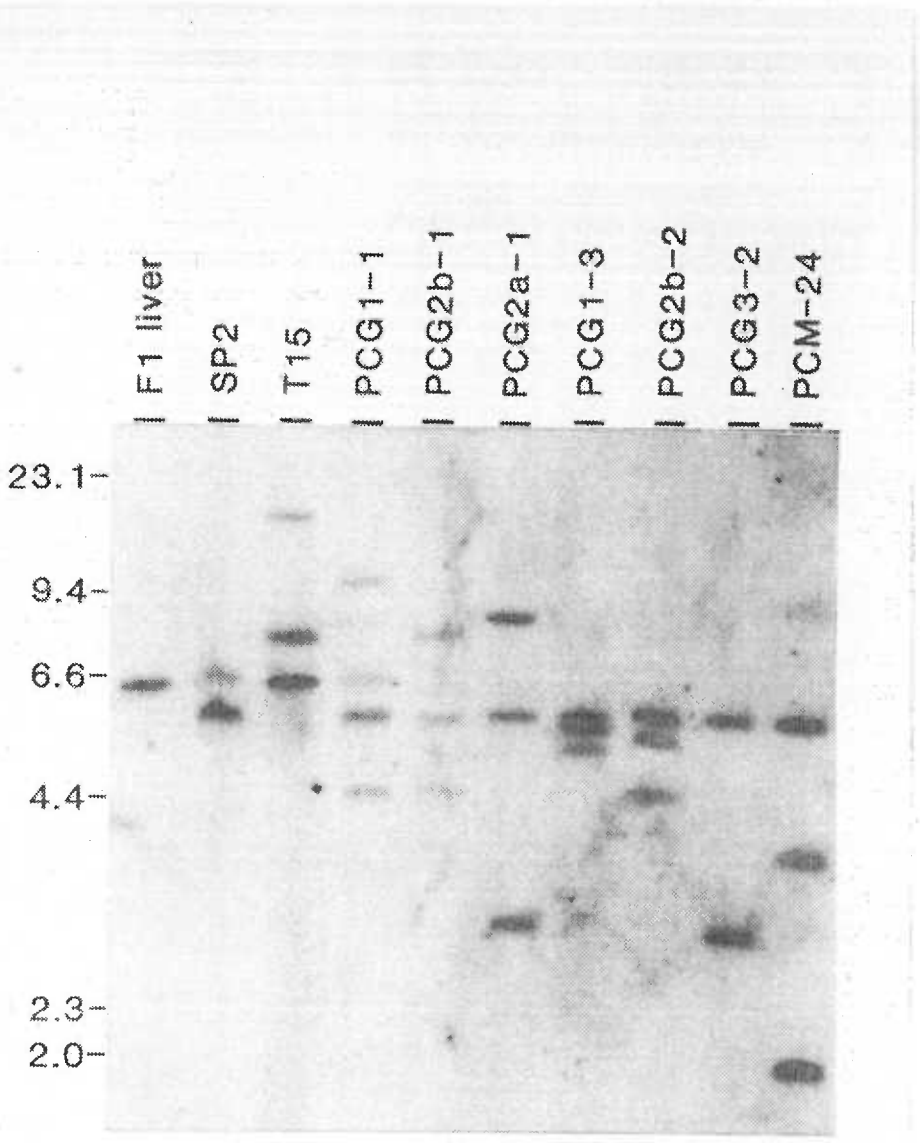

F1 liver
SP2
T15
PCG1-1
PCG2b-1
PCG2a-1
PCG1-3
PCG2b-2
PCG3-2
PCM-24
23.1
9.4
6.6
4.4
2.3
2.0

Table I. Properties of CBA/N X BALB/c F1 Hybridoma Proteins.

Hybridoma	Isotype	Specificity Group	I_{50}[a] NPPC/PC	R36A binding	Idiotype T15[b] (private)	Idiotype T15-VH[c]	L Chain[d]	V1 Gene Rearrangement
PCM24	μ	I	9.5	-	-	-	κ24	-
PCG2a-1	γ2a	II	<0.05	-	-	-	κ1-3	-
PCG1-3	γ1	II	<0.46	-	-	-	κ(24)	-
PCG2b-2	γ2b	II	<0.04	-	-	-	κ(8)	-
PCG3-2	γ3	II	<0.0003	-	-	-	λ2	-
PCG2b-1	γ2b	III	-[e]	-	-	-	κ(24)	-
Myeloma								
TEPC 15	α	I	8.6	+	+	+	κ22	+
M511	α	I	0.2	+	-	+	κ24	+
M603	α	I	7.8	+	-	+	κ8	+

Table I (cont.)

a. NPPC/PC ratio of I_{50} values (I_{50} = mM concentration of PC or NPPC required for 50% inhibition of binding of antibody to PC-histone); data taken from Figure 1.

b. Expression of T15 private idiotopes as defined by the monoclonal antibodies AB1-2 and F6.3; data taken from Figure 2 and references 14, 15.

c. Expression of T15 family V_H region as indicated by binding to the monoclonal antibody TC54; data taken from Figure 2 and references 5,13.

d. Light chains in () are putative L chain assignment. Data taken from reference 6.

e. Although PCG2b-1 binds specifically to PC-protein, it has no measurable affinity for free PC or NPPC as judged by hapten inhibition in an ELISA.

Paper 2

Immunologic Memory to Phosphocholine. X. Participation of the Q52 V_H Gene Family

ABSTRACT

BALB/c mice immunized with phosphocholine-conjugated keyhole limpet hemocyanin (PC-KLH) respond with two major groups of anti-PC antibodies that differ with respect to fine specificity and idiotype. Group I antibodies predominantly bear the T15 idiotype, and show appreciable affinity for the haptens PC and nitrophenyl PC (NPPC) whereas Group II antibodies have appreciable affinity for NPPC only and are T15 idiotype negative.

Previous studies indicated that Group II binding characteristics may derive from the use of novel V gene segments not observed in Group I antibodies. To determine the nature of V_H gene usage in the Group II anti-PC response, we have examined the V_H region of a prototype Group II hybridoma, PCG1-1. The nucleotide sequence obtained from the VDJ region indicates that PCG1-1 utilizes a V_H gene not previously observed in the PC response, one that belongs to the Q52 V_H gene family. The PCG1-1 V_H nucleotide sequence shares 97% identity with the myeloma M141 V_H gene. In addition, PCG1-1 utilizes a D segment most closely related to DSP2.6 rearranged to J_H-3. These data indicate that M141, a V_H gene not seen in Group I anti-PC antibodies, can be utilized to generate a Group II PC-binding antibody. PCG1-1 has been shown previously to express the V_κ1-3 light chain; a characteristic shared by several Group II hybridomas. Furthermore, we examined the V_H gene rearrangements in four λ_1 bearing Group II PC hybridomas that share a common J_H rearrangement with PCG1-1 by Southern blot analysis. A V_H-specific probe that detects M141 V_H rearrangements revealed that all four λ_1 hybridomas as well as PCG1-1 share an identical V_H gene rearrangement to J_H-3. Thus, the M141 V_H gene product is able to utilize two distinct light chains to generate Group II-like PC combining sites.

INTRODUCTION

The primary antibody response to phosphocholine (PC) in BALB/c mice is highly restricted with respect to idiotype expression and variable region gene usage (1-3). A single V_H and V_L combination (V_H-1, V_K22) defines the T15 idiotype which may comprise as much as 95% or more of the initial anti-PC response (4,5). Although the primary response is viewed as restricted, limited heterogeneity exists due to the presence of non-T15 clones that utilize the T15 V_H gene product, V_H-1, but are associated with V_K8 or V_K24 light chains rendering them T15 idiotype negative.

In contrast to the pauciclonal character of the primary response, molecular analysis of the memory response reveals a diverse genetic repertoire to PC. We have described the appearance of new V_L and V_H genes not previously observed in the primary response as well as the continued presence of the antibodies that predominate in the primary response (6-10). Antibodies of the memory response segregate into two major groups based on hapten-binding characteristics (11). Group I antibodies have high affinity for the PC hapten and are predominantly associated with IgM, IgG3 and IgA. These group I antibodies primarily utilize the T15 V_H gene (V_H-1) in association with V_K22, V_K24, and V_K8 and, as indicated above, account for virtually the entire primary response. Group II antibodies differ from Group I in that they bind nitrophenyl PC with greater avidity than PC, are T15 idiotype negative, and predominate the IgG1 and IgG2 isotypes. Unlike Group I antibodies, Group II antibodies are virtually absent in the primary response but can represent 50% of the serum anti-PC antibodies found in the memory response (12). To investigate the molecular basis for differences in

binding specificities between the two groups of PC antibodies, B-cell hybridomas generated from an anti-PC memory response were analyzed with respect to V_H and V_L gene utilization (6-10). Isoelectric focusing patterns revealed V_L genes not previously observed in Group I antibodies, in particular the V_κ1-3 gene product was used by several Group II antibodies (7,8). In addition, one hybridoma was found to utilize λ_2 while others expressed light chains that are yet unidentified or that have been observed previously in Group I antibodies (8). Thus, the altered binding specificity found in Group II hybridomas may be due in part to the expression of novel light chains. V_H gene analysis thus far has revealed that genes distinct from the T15 gene family are expressed by Group II hybridomas although their identity was not determined (9,10); partial amino acid sequence analysis of two hybridomas suggested that some Group II antibodies utilize a V_H gene most closely related to the V_H-12 gene product (10).

In the present work we explored the Group II anti-PC repertoire by nucleotide sequencing the V_H gene expressed by a prototype Group II hybridoma previously shown to utilize the V_κ1-3 light chain gene, a feature common to several Group II hybridomas. Sequence analysis indicates that the hybridoma utilizes the myeloma M141 gene (13) which is a member of the Q52 V_H gene family (14). The use of the M141 V_H gene in a PC-binding antibody has not been previously observed. Interestingly, no other Group II V_κ1-3 bearing hybridoma was shown to utilize the M141 gene. However, four Group II hybridomas that utilize λ_1 light chains were found to express the M141 gene rearranged to the same J_H segment (J_3) as that observed in the PCG1-1 hybridoma expressing the V_κ1-3 light chain.

These results indicate that the M141 V_H gene may associate with two distinct light chains to yield combining sites with Group II binding characteristics. This finding extends our earlier observations that increased antibody heterogeneity occurs during the development of immunologic memory to PC, and that the expression of new V gene combinations may contribute substantially to this process.

MATERIALS AND METHODS

Immunization and Preparation of Hybridomas.

Balb/c mice were immunized with PC-KLH coupled to bentonite (hybridomas PCG1-1 (6), PCG3-3 (15), and PCG2b-4 (15)) or adsorbed to alum (hybridoma PCG2b-3 (15) or in complete Freund's adjuvant (hybridoma aPC-1-56-1, immunization described in ref. 6). Following an i.v. boost of PC-KLH, spleen cells were removed and fused with the myeloma SP2/0 as described (6,15).

Southern Blot Analysis.

Genomic DNA was isolated from cells grown in tissue culture or, in the case of M141 and T15, from subcutaneous tumors grown in BALB/c mice. High molecular weight DNA was prepared by the guanidinium thiocyanate/cesium chloride method (16) or by the method of Blin and Stafford (17). 10 μg of EcoR1-digested DNA was electrophoresed (0.7% agarose) for 16 hr and transferred to nylon membranes (Biotrans, ICN, Irvine, CA) according to Southern (18). Prehybridizations and hybridizations were performed for 16 hr at 42°C in 6X SSC (1X SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 5X Denhardt's solution (1X Denhardt's = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 50% formamide, 10% dextran sulfate, 0.01 M EDTA, and 200 μg/ml salmon sperm DNA. Filters were washed according to Brodeur and Riblet (14) at 55°C (J_H probe) or 65°C (5' flanking and V_H coding region probes) for 30 min in 3X SSC, 5 mM EDTA, 0.1% SDS, 5X Denhardt's and 25 μg/ml salmon sperm DNA followed by three high stringency washes (30 min) at 55°C or 65°C in 0.2X SSC, 5 mM EDTA, 0.1% SDS and 25 μg/ml salmon sperm DNA. To remove the probe completely prior to rehybridization, the membrane was washed at

65°C for 60 min in 10 mM sodium phosphate (pH 6.5) and 50% formamide followed by one wash at room temp for 15 min in 2X SSC. DNA probes were labeled by random primer extension (19) to a specific activity of 0.5-1.0 x 10^9 dpm/μg and hybridizations were performed using 0.5-1.0 x 10^8 dpm/membrane. A 3.2 kilobase pair (kbp) probe containing all four BALB/c J_H genes cloned into pBR322 (20) was provided by Drs. R. Perlmutter and L. Hood. The PCG1-1 V_H coding region probe is a 620 base pair (bp) Hind III-Pst I fragment containing the V_H coding region and J_H-3. The PCG1-1 5' flanking region probe covers a region located 129 base pairs 5' of the leader sequence and extending 1.3 kbp upstream of the V_H region.

Isolation of Recombinant Bacteriophage and J_H Fragment Cloning.

EcoR1 digested PCG1-1 DNA was ligated to λ gt Wes B EcoR1 arms (Bethesda Research Laboratories, Gaithersburg, MD.) and packaged *in vitro* (Packagene, Promega Biotec, Madison, WI). Approximately $9x10^5$ phage recombinants were screened according to Maniatis *et al.* (21) for J_H-containing sequences using a J_1-J_4 specific probe (20). A J_H positive recombinant found to contain the 9.5 kbp EcoR1 J_H insert was selected for sequence analysis. The 9.5 kbp EcoR1 J_H insert was subcloned into pBR325 (22) for restriction enzyme analysis and a 3.0 kbp Xba I J_H fragment was further subcloned into pUC 18 (23).

DNA Sequence Analysis.

The VDJ region that lies within a 620 bp Hind III-Pst I fragment was sequenced by the dideoxynucleotide chain termination method (24) using the M13 mp18/mp19 vector system (23). DNA sequence comparisons were provided by Bionet™ National Computer Resources.

RESULTS

V_H gene cloning and nucleotide sequence analysis.

The Group II hybridoma, PCG1-1, has three JH rearrangements; 9.5, 5.7 and 4.4 kbp EcoR1 fragments (Fig. 1a). Only the 9.5 kbp band was expected to contain the productive VDJ rearrangement utilized by the B cell since the 5.7 kbp band can be attributed to the SP2/0 fusion partner and the 4.4 kbp band contains an unusual rearrangement rendering it nonproductive (Stenzel-Poore and Rittenberg, submitted for publication). To isolate the productive VDJ allele a hybridoma DNA library comprised of EcoR1 cut PCG1-1 DNA ligated into λ Wes B was screened for J_H-containing recombinants using a J_H-specific probe. A J_H-positive recombinant with the 9.5 kbp insert was identified and the insert subcloned into pBR325 for restriction mapping (Fig. 1b). A 3.0 kbp Xba I fragment containing the VDJ rearrangement was subcloned into pUC 18 (Fig. 1c).

The complete nucleotide sequence of the VDJ region was obtained using the dideoxynucleotide sequencing method (24). The 620 base pair sequence is shown in Figure 2. The J region is identical to J_H-3 (25) and represents a departure from the nearly universal usage of J_H-1 reported for other PC-specific antibodies (1). Notable exceptions include a CBA/N anti-PC hybridoma that utilizes J_H-4 in combination with the T15 V1 gene (26) and a CBA/J hybridoma expressing a member of the S107 (T15) gene family, V11 rearranged to J_H-3 (27,28). The 16 bp D region is most closely related to DSP2.6 exhibiting only two nucleotide differences from the published germline sequence (29). The use of this D segment is uncommon for PC-binding antibodies since the majority use DFL16.1 (1).

The V_H region shares 97% homology with the V_H gene expressed by the the myeloma, M141, and exhibits even fewer differences (9 nucleotide changes, 98% homology) from the germline homologue (PJ14) of the V_H gene from M141 (13). The most striking feature is the paucity of changes in the hypervariable regions of PCG1-1. Of the nine differences occurring between the PCG1-1 V_H sequence and the M141 germline gene (PJ14), four changes occur in noncoding segments, either in the 5' flanking region or in the intervening sequence following the leader segment. Of the five coding region changes, only one occurs in the hypervariable region, at position 31, resulting in a glycine to valine interchange. The remaining four substitutions occur in the framework region (Val36→Ile, Lys75→Gln), two of which represent silent changes.

M141 V_H gene is rearranged in PCG1-1.

It has been reported that individual members within a closely related gene family may differ by only a few nucleotides (30) making it difficult to determine whether the sequence of PCG1-1 is derived by somatic mutation from the PJ14 germline gene or whether the observed differences are due to the use of another V_H family member. In addition, PJ14 is a member of the relatively complex V_H family-Q52, which contains ∿15 V_H genes (14). Southern blot analysis of EcoRI digested PCG1-1, liver and SP2/0 DNA probed with the rearranged PCG1-1 V_H coding region illustrates this complexity (Fig. 3). It was not possible to determine if a rearrangement of PJ14 had occurred because of multiple bands in the region of interest. Although the band complexity made it difficult to detect a rearrangement, a doublet is present at ∿9.5 kbp that is not seen in SP2/0 or liver DNA.

To determine more accurately if the V_H rearrangement in PCG1-1 is derived from the M141 gene, we used the V_H 5' flanking region (Fig. 1d) from the cloned PCG1-1 rearrangement which detects only a single member of the Q52 family in germline DNA (Fig. 4b, lane 1). If both PCG1-1 and the myeloma M141 shared similar rearrangements it would indicate that they both employ the same V_H gene. Figure 4a is a Southern blot of EcoRI digested DNA probed with J_H. M141 shows a J_H rearrangement at ~9.0 kbp and PCG1-1 shows a rearrangement at 9.5 kbp. M141 is known to utilize J_H-4 while PCG1-1 uses J_H-3. The difference in size (~500bp) between the two J_H bands is consistent with the difference in J_H segment usage. The J_H probe was removed by washing and the blot was reprobed with the V_H 5' flanking region to determine whether M141 exhibited the same V_H rearrangement as PCG1-1 and whether the rearrangement detected was the same as that observed with the J_H probe. Figure 4b shows that the 5' flanking probe detects 9.5 and 9.0 kbp rearrangements in PCG1-1 and M141 respectively. The same rearranged bands were also detected using the J_H probe suggesting both the hybridoma and the myeloma utilize the same germline V_H gene. In addition, the 5' flanking probe also detects a common band in liver DNA and in PCG1-1, M141 and SP2/0 DNA reflecting the germline configuration of PJ14(M141).

Thus, it appears that the V_H gene expressed by PCG1-1 is derived from the germline gene of M141, PJ14, and that the observed nucleotide differences reflect somatic changes that presumably occurred during an antigen-driven response.

PCG1-1 heavy chain associated with a λ light chain: analysis of V_H and J_H rearrangements.

The Group II binding characteristics of PCG1-1 are attained through the use of the M141 V_H gene and the V_K1-3 light chain. Four additional hybridomas were found to share the same V_H and J_H rearrangement as PCG1-1 yet differed in that they used the λ_1 light chain, which itself is an unusual feature for anti-PC antibodies. Southern blot analysis of four Group II hybridomas that utilize the λ_1 light chain (see accompanying manuscript ref. 15) is shown in Fig. 4. The blot was first probed with J_H to detect J_H rearrangements and then washed to remove the J_H probe before re-probing with the PCG1-1 5' flanking probe to detect the unique rearrangement of the M141 germline gene. All four λ_1 hybridomas and the V_K1-3 bearing hybridoma PCG1-1 share a 9.5 kbp band with both probes. These data indicate that the M141 V_H gene can associate with either κ- or λ-light chains to generate PC binding antibodies with Group II-like specificity. Furthermore, the finding that all five hybridomas appear to use the same V_H rearranged to J_H-3 suggests that this association may be important in antigen recognition by such antibodies belonging to Group II.

Properties of anti-PC hybridomas that utilize the M141 V_H gene.

A summary of the 5 hybridoma antibodies analyzed in this study is presented in Table I. The antibodies represent four separate fusions of BALB/c spleen cells following hyperimmunization with PC-KLH. The hapten-binding characteristics of these Group II antibodies have been described in detail elsewhere (15). T15 is included for illustration and comparison of binding properties characteristic of the Group I-like specificity. The results indicating light chain usage by Group II

antibodies are derived from a previous study (7) and the accompanying report (15).

DISCUSSION

We have identified a new V_H gene that can be utilized by antibodies to phosphocholine. The identifiction of this V_H gene confirms our previous observations that novel V_H genes not apparent in the primary response are employed and represent a substantial contribution to the diversity seen in the memory response to PC-KLH. A minimum of three V_H gene families (Q52, S107, J558 (10)) thus contribute to the heterogeneity of the PC response. The M141 V_H gene utilized by the hybridoma PCG1-1 is found in combination with the V_K1-3 gene product which represents a V_L gene also not found in the primary response. Thus, the heavy and light chains that form the combining site of this Group II antibody derive from new V gene products found mainly in the memory response.

Additional diversity is provided by the D and J segments used in PCG1-1. The use of J_H-3 has been observed in a single report of a PC-binding antibody of CBA/J origin (27,28). Although the diversity of D regions found in the PC response implies little or no direct role in PC binding (1,3), it has been suggested that the overall length of the D segment may be important in influencing the structure of the binding site (31). The majority of PC antibodies reported thus far utilize a D segment five amino acids long. The D region of PCG1-1 also consists of five amino acids and the sequence Tyr.Tyr.Arg.Tyr.Asp is similar to that of several Group I PC antibodies (1). The germline gene most closely related to the D region of PCG1-1 is derived from the DSP2 family, DSP2.6 (29). The two sequences differ by two nucleotide substitutions that result in a change from the small neutral amino acid glycine to the very large, positively charged arginine. It is not clear from a single case such as this whether the substitution confers a meaningful change

involving antigen binding; it does suggest however, that D region length alone is not sufficient for strong PC binding since PCG1-1 has a relatively low avidity for free PC yet shares a similar D region with other hybridomas, of which at least one binds PC with high avidity (32). It would be of interest to assess D region usage of the anti-PC hybridomas that utilize the λ_1 light chain to determine whether a common sequence exists between these Group II hybridomas. Preliminary results obtained from a partial nucleotide sequence of Group II hybridoma, PCG2b-4, indicate that it expresses a D region distinct from PCG1-1 (Stenzel-Poore and Rittenberg, unpublished). The D-J junction of PCG2b-4 also differs in that the J_H-3 segment is truncated by three amino acids in this λ_1-bearing hybridoma. It is possible that while the two hybridoma proteins are able to generate Group II-like binding specificities using the same V_H and J_H genes that differences in the third hypervariable region (D-J junction) may be necessary to accomodate the use of the two distinct light chains.

The structural basis for antigen binding by PCG1-1 is not yet clear. The immunizing form of the antigen is PC coupled to protein via a diazophenyl linker. Group II antibodies have a much higher avidity for the nitrophenyl form of PC (NPPC) than for PC itself (11). The phenyl ring structure may play a dominant role in antibody binding even though Group II antibodies require the PC moiety as well (11). It is interesting that several antibody responses to phenyl-containing hapten-protein conjugates utilize V genes similar or identical to those expressed by PCG1-1. The hapten 2-phenyl-5-oxazolone (phOX) bears limited structural homology to NPPC (33) and some antibodies generated in response to phOX coupled to protein also use a V_H gene highly homologous

to M141 (34) and the light chain V_K1-3 is commonly observed in anti-phOX antibodies (35,36) although the M141 V_H and V_K1-3 combination has not been observed. The specificity of M141 itself is unknown (37); we have found that it does not bind to PC-histone in the ELISA (data not shown). In addition, the myeloma MOPC 460 which recognizes the dinitrophenyl hapten also utilizes a member of the Q52 V_H family and the V_K1-3 light chain (37). Thus, it is quite possible that the phenyl ring provides a common feature in each of these haptens that is particularily well accomodated by antibodies generated through use of these two gene families. It is clear, however, that other combinations such as the M141 V_H gene associated with λ_1 also form an antibody combining site capable of binding NPPC. Sequence analysis of the V_H and V_L region utilized by the anti PC λ-bearing hybridomas described in this report may point to conserved features required for binding that are shared by these hybridomas.

In order to evaluate the structure of the V_H region of PCG1-1 in terms of its unusual PC-binding specificity (i.e., high avidity for NPPC but low avidity for PC) we utilized the structure of the PC-binding myeloma protein-McPC 603 (M603) which has been well-studied by x-ray crystallography (38,39). The crystal structure of M603 has served not only as a model for anti-PC proteins (39,40) but also for antibodies of other specificities (41-43). Differences in fine specificity that would allow appreciable binding of the nitrophenyl derivative of PC and only weak binding to PC itself as is the case with PCG1-1 (in contrast with M603 where the reverse is true, i.e. I_{50}, NPPC/PC = 9.5 (11)) may become apparent through examining the amino acids at the critical hapten contact residues as defined by M603. We reasoned that there may be shared amino

acid residues that enable both proteins to bind choline but that differences may be seen in the phosphate-binding subsite.

The three dimensional structure of M603 has been resolved to the 2.7 Å level (38,39) and in combination with sequence data of PC-binding antibodies (39,40) provides useful information regarding the structural basis for the specificity of binding of antibodies to antigen. Crystallographic analysis of M603 with PC in the active site (39,40) has revealed a pocket-like site for binding PC, accommodating the positively charged trimethylammonium portion of the choline moeity in the bottom of the pocket and the negatively-charged phosphate on the surface near the front of the pocket. Accumulation of numerous sequences encoding anti-PC antibodies similar to M603 has enabled the assignment of essential amino acid positions for compatible V_H and V_L interactions that form the deep pocket-binding site and electrostatic interactions and hydrogen bonding between the hapten and amino acid side chains (39). At the front of the pocket of M603 there are two hydrogen bond donors that interact with the phosphate oxygen; these are heavy chain residues Tyr33H and Arg52H. These residues and possibly Lys52bH contribute to the heavy chain contacts in the phosphate-binding subsite (39,40). Residues that could serve a similar function in the corresponding V_H region of PCG1-1 appear to be missing. The glycine at position 33H in PCG1-1 does not offer the phenolic-OH of the tyrosine present in M603 that is considered to be used in hydrogen bonding one oxygen of the phosphate. Arg52H, that is also thought to form a hydrogen bond with another oxygen of the phosphate (39,40) is replaced by a bulky tryptophan residue completely lacking a polar group. It is difficult to evaluate the residue corresponding to Lys52bH of M603 since the CDR2 region in which this residue lies is

shorter in PCG1-1 than in M603; nevertheless it can be seen that of the six residues flanking the position corresponding to Lys52bH none contains a positive charge. There are two negatively charged residues, Glu35H and Glu58H (using the Kabat numbering system (37) and taken from ref. 39) in the active site of M603 that have been shown to interact with the positively charged choline moiety (39,40). Although it has been suggested that Glu35H may be too far away for direct contact it would provide an electrostatically favorable environment for choline binding (44). Glu35H has been shown to form a VL contact with Tyr94L and thus may be important in stablizing the conformation of the combining site (44). These heavy chain residues and possibly Asn95H line the bottom of the pocket and form the subsite for choline binding (39,40). Using M603 as a model for the active site of PCG1-1 (although recognizing that the crystal structure of the latter is unknown) it appears that an analogous choline-binding site could also exist in the PCG1-1 V_H sequence. The PCG1-1 V_H residues corresponding to the M603 choline subsite (Fig. 5) generate a net negative charge that may permit choline binding. The residues corresponding to M603 Glu35H, Glu58H and Asn95H exist in PCG1-1 as Asn35H, Asp58H and Ala95H. Assuming the appropriate geometry, the negatively charged Asp residue would be a most reasonable target for interacting with the postively charged trimethylammonium moiety.

Thus, while there is a conservation of a net negative charge in what may be the contact subsite for choline, weaker interactions or different essential residues would have to interact with the phosphate moiety if the analogy with M603 were to hold. It has been suggested that Asp residues at positions 95H and 97H and Trp at 100aH are also important features affecting phosphocholine-binding sites of some proteins (40).

It was proposed that the Asp residues are electrostatically favorable for choline binding thus explaining the low affinity for this ligand by M603 which has an Asn at position 95H and Tyr at 97H (Fig. 5b). Moreover it was suggested that Trp100aH, although not directly involved in binding, contributes to phosphate binding by partially shielding the latter from a negative electrostatic interaction with Asp95H; thus it was possible to explain the reduced affinity for PC of M167 since the latter has a Gly in place of Trp100aH. Although PCG1-1 has the Trp100aH residue it lacks Asp at both positions 95H and 97H which would explain its failure to bind choline detectably. This feature together with the lack of an obvious phosphate-binding subsite in PCG1-1 as discussed above could explain its weak affinity for PC except when it is conjugated to protein thus placing more emphasis on a site in which the phenyl ring can play an important role. Aromatic residues that could conceivably interact with the phenyl ring are located at CDR positions Tyr32H, Trp52H and Tyr59H.

The interactions of light chain residues are not considered here since there is no information on this V_K1-3 sequence beyond residue 23 (7). However, the fact that the V_K1-3 light chain has been observed in association with several Group II hybridomas (7,8) suggests that it may play an active but not essential role in determining the character of the hapten-binding site (V_K1-3 cannot be essential since antibodies with the same fine specificity pattern can also be generated with λ-light chains (15 and Table I).

The memory response to PC displays a level of genetic complexity not seen in the primary response. It is clear from this work and our previous findings that some Group II antibodies (6,7,10,11) observed in the memory response have their origins in novel gene usage and novel gene

combinations that are not detected during the initial stages of the immune response. In this respect maturation of the immune response to PC resembles that seen in the responses to phOX (34-36) and to (4-hydroxy-3-nitrophenyl)acetyl (NP) (45) and stands in contrast to maturation of the arsanilate response in which more genes appear to be expressed in the primary than in the memory response (46-49). Although the extent of the genetic heterogeneity expressed in the memory response to PC-KLH is not yet clear, preliminary evidence for additional V_H genes has been obtained (Stenzel-Poore and Rittenberg, unpublished) and additional light chains have been noted in serum antibodies (8). Thus, the anti-PC response continues to provide a useful model of immune responsiveness to simple determinants and as the genes involved are revealed the model may provide a basis for deriving the rules governing their selection.

Acknowledgements

We thank P. Stenzel and N. Keller for valuable technical assistance, M. Potter for providing the MOPC 141 tumor cell line, M. Brown, U. Bruderer, A. Buenafe, F. Makowski and T. Tittle for helpful discussions, and J. Hoff for typing the manuscript.

REFERENCES

1. Perlmutter, R.M., S.T.Crews, R. Douglas, G. Sorenson, N. Johnson, N. Nivera, P.J. Gearheart, and L. Hood. 1984. The generation of diversity in phosphorycholine-binding antibodies. Adv. Immunol. 35:1.

2. Claflin, J.L. and J.M. Davie. 1974. Clonal nature of the immune response to phosphorycholine. IV. Idiotypic uniformity of binding site associated antigenic determinants among mouse anti phosphorycholine antibodies. J. Exp. Med. 140:673.

3. Perlmutter, R.M. in The Biology of Idiotypes, Plenum Press, New York, 1984 p. 59.

4. Claflin, J.L., and S. Rudikoff. 1977. Uniformity in a clonal repertoire: A case for a germline basis of antibody diversity. Cold Spring Harbor Symp. Quant. Biol. 40:725.

5. Cosenza, H., and H. Kohler. 1972. Specific inhibition of plaque formation to phosphorycholine by antibody against antibody. Science. 176:1027.

6. Chang, S.P., R.M. Perlmutter, McK. Brown, C.H. Heusser, L. Hood, and M.B. Rittenberg. 1984. Immunologic memory to phosphocholine. IV. Hybridomas Representative of Group I (T15-like) and Group II (non-T15-like) antibodies utilize distinct V_H genes. J. Immunol. 132:1550.

7. Todd, I., S.P. Chang, R.M. Perlmutter, R. Abersold, C.H. Heusser, L. Hood, and M.B. Rittenberg. 1984. Immunologic memory to phosphocholine. V. Hybridomas representative of group II antibodies utilize V_K1-3 gene(s). J. Immunol. 132:1556.

8. Todd, I., McK. Brown, and M.B. Rittenberg. 1985. Immunologic memory to phosphorycholine. VI. Heterogeneity in light chain gene expression. Eur. J. Immunol. 15:177.

9. Brown, McK., M. Stenzel-Poore, and M.B. Rittenberg. 1985. Immunologic memory to phosphocholine. VII. Lack of T15 V1 gene utilization in Xid anti-PC hybridomas. J. Immunol. 135:3558.

10. Rittenberg, M.B., R.W. Glanville, R.H. Abersold, S.P. Chang, and McK. Brown. 1986. Immunologic memory to phosphorycholine (PC). VIII. Expression of the V_H-12 gene product in the response to PC-keyhole limpet hemocyanin. Eur. J. Immunol. 16:503.

11. Chang, S.P., McK. Brown, and M.B. Rittenberg. 1982. Immunologic memory to phosphorycholine. II. PC-KLH induces two antibody populations that dominate different isotypes. J. Immunol. 128:702.

12. Chang, S.P., McK. Brown, and M.B. Rittenberg. 1982. Immunologic memory to phosphorycholine. III. IgM includes a fine specificity population distinct from TEPC 15. J. Immunol. 129:1559.

13. Sakano, H., R. Maki, Y. Kurosawa, W. Roeder, and S. Tonegawa. 1980. Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy chain genes. Nature 286:676.

14. Brodeur, P.H. and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse. I. One hundred Igh-V genes comprise seven families of homologous genes. Eur. J. Immunol. 14:922.

15. Rittenberg, M.B., T.J. Hall, M. Stenzel-Poore. 1986. Accompanying manuscript. J. Immunol.

16. Mantiatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory) p. 196.

17. Blin, N. and D.W. Stafford. 1976. A general method for the isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. 3:2303.

18. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503.

19. Feinberg, A. and B. Vogelstein. 1982. A technique for radio labeling DNA restriction endonuclease fragments to high specific activity. Analyt. Biochem. 132:6.

20. Calame, K., J. Rogers, P. Early, M. Davis, D. Livant, R. Wall, and L. Hood. 1980. Mouse C_u heavy chain immunoglobulin gene segment contains three intervening sequences separating domains. Nature 284:452.

21. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory) p. 320.

22. Prentki, P., F. Darch, S. Iida, and J. Meyer. 1981. The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. Gene 14:289.

23. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC 19 vectors. Gene 33:103.

24. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. USA 74:5463.

25. Gough, N.M. and O. Bernard. 1981. Sequences of the joining region genes for immunoglobulin heavy chains and their role in generation of antibody diversity. Proc. Natl. Acad. Sci. 78:509.

26. Clarke, S.H., J.J. Kenny, D.G. Sieckmann, and S. Rudikoff. 1984. Amino acid sequence of a phosphocholine-binding antibody from an immune defective CBA/N mouse employing the T15 V_H region associated with unusual D_H, J_H and V_K segments. J. Immunol. 132:1544.

27. Clarke, S.N, and S. Rudikoff. 1984. Evidence for gene conversion among immunoglobulin heavy chain variable region genes. J. Exp. Med. 159:773.

28. Clarke, S.H., J.L. Claflin, and S. Rudikoff. 1982. Polymorphisms in immunoglobulin heavy chains suggesting gene conversion. Proc. Natl. Acad. Sci. USA 79:3280.

29. Kurosawa, Y. and S. Tonegawa. 1982. Organization, structure and assembly of immunoglobulin heavy chain diversity DNA segments. J. Exp. Med. 155:201.

30. Bothwell, A.L.M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1981. Heavy chain variable region contribution to the NP^b family of antibodies: somatic mutation evident in a γ2a variable region. Cell 24:625.

31. Clarke, S.J., J.L. Claflin, M. Potter, and S. Rudikoff. 1983. Polymorphisms in anti-phosphocholine antibodies reflecting evolution of immunoglobulin families. J. Exp. Med. 157:98.

32. Gearhart, P., N.D. Johnson, R. Douglas, and L. Hood. 1981. IgG antibodies to phosphorycholine exhibit more diversity than their IgM counterparts. Nature 291:20.

33. Gell, P.G.H., C.R. Harington, and R.P. Rivers. 1946. The antigenic function of simple chemical compounds: production of precipitans in rabbits. Brit. J. exp. Path. 27:19.

34. Kaartinen, M., G.M. Griffiths, A.F. Markham, and C. Milstein. 1983. mRNA sequences define an unusally restricted IgG response to 2-phenyloxazolone and its early diversification. Nature 304:320.

35. Berek, C., M. Griffiths, and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. Nature 316:412.

36. Kaartinen, M., J. Pelkonen, and O. Mäkelä. 1986. Several V genes participate in the early phenyloxazolone response in various combinations. Eur. J. Immunol. 16:98.

37. Kabat, E.A., T.T. Wu, H. Bilofsky, M. Reid-Miller and H. Perry. 1983. Sequences of proteins of immunological interest. U.S. Department of Health and Human Services, National Institutes of Health, Bethesda.

38. Satow, Y., G.H. Cohen, E.A. Padlan, and D.R. Davies. 1986. Phosphocholine binding immunoglobulin Fab McPC603, and x-ray diffraction study at 2.7 Å. J. Mol. Biol. 190:593.

39. Padlan, E.A., G.H. Cohen, and D.R. Davies. 1985. On the specificity of antibody/antigen interactions: phosphocholine binding to McPC603 and the correlation of three dimensional structure and sequence data. Ann. Inst. Pasteur. Immunol. 136C:271.

40. Padlan, E.A., D.R. Davies, S. Rudikoff, and M. Potter. 1976. Structural basis for the specificity of phosphorycholine-binding immunoglobulin. Immunochemistry 13:945.

41. Padlan, E.A., D.R. Davies. I. Pecht, D. Givot, and C. Wright. 1976. Model-binding studies of antigen-binding sites: The hapten-binding site of MOPC-315. Cold Spring Harbor Symp. Quant. Bio. 41:627.

42. de la Paz, P., B.J. Sutton, M.J. Darsley, and A.R. Rees. 1986. Modelling of the combining sites of three anti-lysozyme monoclonal antibodies and of the complex between one of the antibodies and its epitope. Embo. J. 5:415.

43. Chothia, C., A.M. Lesk, M. Levitt, A.G. Amit. R.A. Mariuzza, S.E.V. Phillips, nad R.J. Poljak. 1986. The predicted structure of immunoglobulin D1.3 and it comparison with the crystal structure. Science 122:755.

44. Rudikoff, S., A.M. Giusti, W.D. Cook, and M.D. Scharff. 1982. Single amino acid substitution altering antigen binding specificity. Proc. Natl. Acad. Sci. USA 79:1979.

45. Boersch-Supan, M.E., S. Agarwal, M.E. White Scharf, and T. Imanishi-Kari. 1985. Heavy chain variable region. Multiple gene segments encode anti-4-(hydroxy-3-nitrophenyl)acetyl idiotypic antibodies. J. Exp. Med. 161:1272.

46. Wysocki, L., T. Manser, and M. Gefter. 1986. Somatic evolution of variable region structures during an immune response. Proc. Natl. Acad. Sci. USA 83:1847.

47. Siekevitz, M., S.Y. Huang, and M. Gefter. 1983. The genetic basis of antibody production: a single heavy chain variable region encodes all molecules bearing the dominant anti-arsonate idiotype in the strain A mouse. Eur. J. Immunol. 13:123.

48. Wysocki, L.J., M. Margolies, B. Huang, D. Nemazee, D.S. Wechsler, V. Sato, J. Smith, and M. Gefter. 1985. Combinatorial diversity within variable regions bearing the predominant anti-p-azophenyl-arsonate idiotype of strain A mice. J. Immunol. 134:2740.

49. Slaughter, C.A., D.J. Jeske, W.A. Kuziel, E.C.B. Milner, and J.D. Capra. 1984. Use of J_H4 joining segment gene by an anti-arsonate antibody that bears the major A-strain cross reactive idiotype but displays diminished antigen binding. J. Immunol. 132:3164.

TABLE I

Properties of BALB/c Group II PC-binding hybridomas that utilize V_H M141[a]

Hybridoma or Myeloma	Isotype	Group	NPPC:PC[b,c]	L Chain	V_H[a]	J_H
PCG1-1	γ_1	II	<0.013	κ1-3	M141	J3
aPC-1-56-1	γ_1	II	<0.0063	λ1	M141	J3
PCG2b-3	γ2b	II	<0.053	λ1	M141	J3
PCG2b-4	γ2b	II	<0.067	λ1	M141	J3
PCG3-3	γ3	II	<0.044	λ1	M141	J3
T15	α	I	8.6	κ22[d]	V1[d]	J1[d]

[a] Germline gene of M141 is referred to as PJ14 (13).

[b] Ratio of I_{50} values for NPPC and PC.

[c] Data taken from ref. 5 and 15.

[d] From ref. 3.

Figure 1a) Southern blot analysis of EcoRI digested genomic DNA hybridized to a J_H probe. Sources of DNA are indicated above each lane. The arrow indicates the productive J_H rearrangement cloned from hybridoma PCG1-1 DNA. L indicates leader segment. Size markers are indicated in kilobase pairs (kbp).

Figure 1b) Restriction map of the cloned 9.5 EcoRI fragment containing the VDJ rearrangement from PCG1-1 DNA. The fragment is shown cloned into the plasmid vector pBR325. Restriction sites are as follows: E, EcoRI; X, Xba I; P, Pst I; (P), Pst I approximate location; H, Hind III.

Figure 1c) J_H subclone containing a 3.0 kbp Xba I fragment cloned into the plasmid vector pUC 18. Restriction sites are as indicated in 1b.

Figure 1d) DNA probes isolated from the cloned V_H coding region (solid box) and 5' flanking region (hatched box) of PCG1-1.

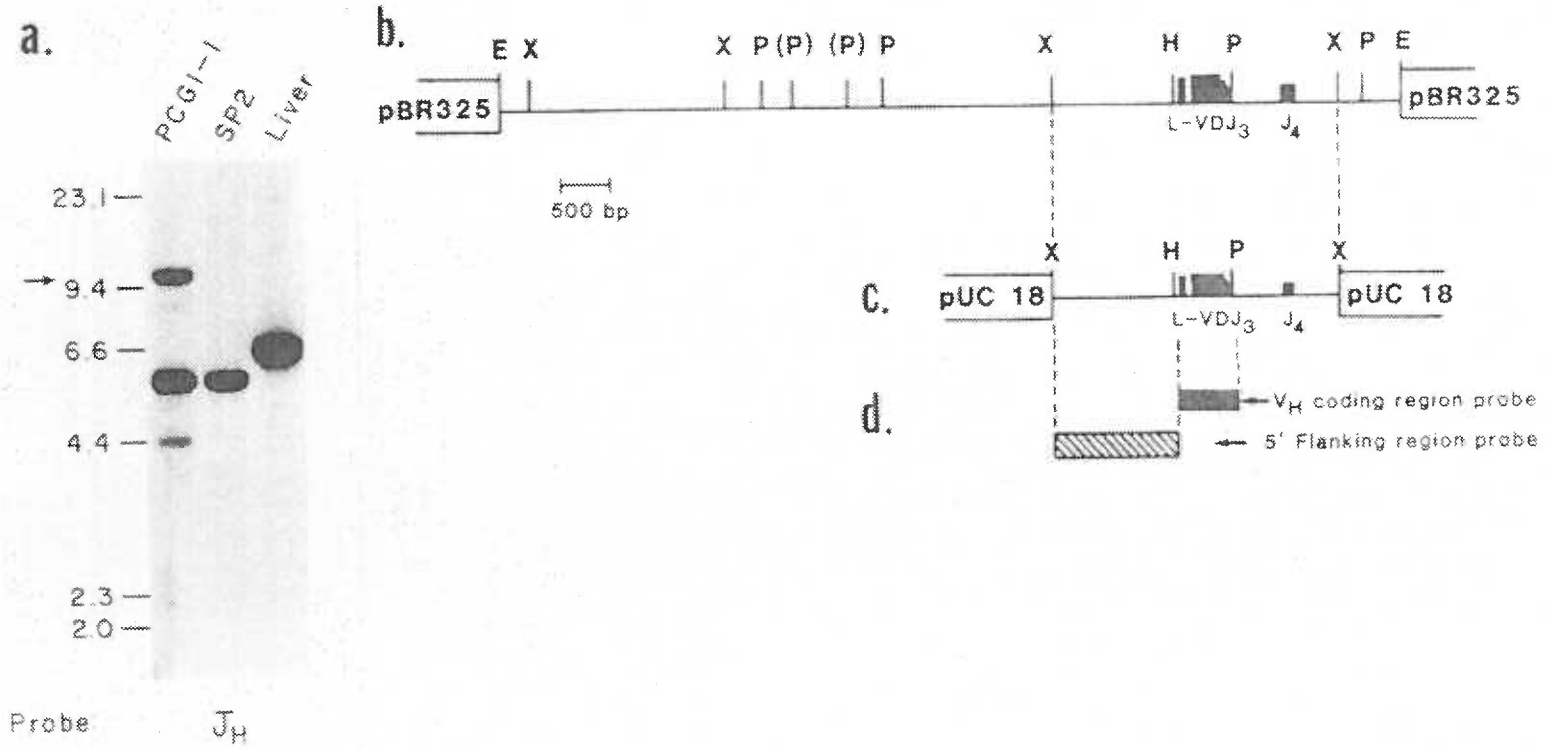

a.
PCG1-1
SP2
Liver
23.1
9.4
6.6
4.4
2.3
2.0
Probe
J_H
b.
E X
X P(P) (P) P
X
H P
X P E
pBR325
pBR325
L-VDJ3
J4
500 bp
C.
X
H P
X
pUC 18
pUC 18
L-VDJ3
J4
d.
V_H coding region probe
5' Flanking region probe

Figure 2) Nucleotide sequence comparison of PCG1-1 V_H and the germline M141 gene - PJ14. Dashed lines indicate sequence identity; asterisks indicate a deletion.

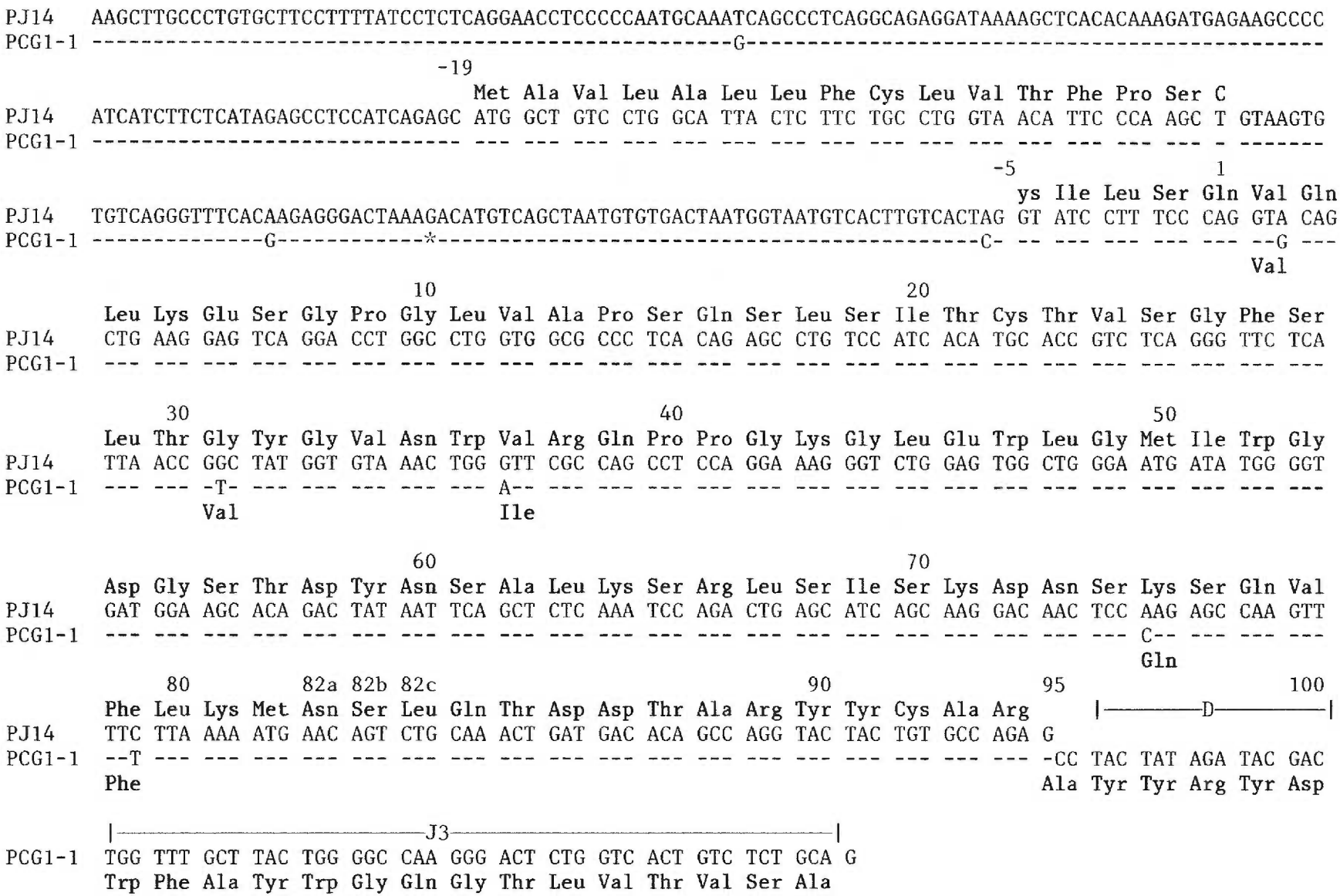

PJ14 AAGCTTGCCCTGTGCTTCCTTTTATCCTCTCAGGAACCTCCCCCAATGCAAATCAGCCCTCAGGCAGAGGATAAAAGCTCACACAAAGATGAGAAGCCCC
PCG1-1 ----------G----------
-19
Met Ala Val Leu Ala Leu Leu Phe Cys Leu Val Thr Phe Pro Ser C
PJ14 ATCATCTTCTCATAGAGCCTCCATCAGAGC ATG GCT GTC CTG GCA TTA CTC TTC TGC CTG GTA ACA TTC CCA AGC T GTAAGTG
PCG1-1 ---------- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- - -------
-5 1
ys Ile Leu Ser Gln Val Gln
PJ14 TGTCAGGGTTTCACAAGAGGGACTAAAGACATGTCAGCTAATGTGTGACTAATGGTAATGTCACTTGTCACTAG GT ATC CTT TCC CAG GTA CAG
PCG1-1 ----------G----------*----------C- -- --- --- --- --- --G ---
Val
10 20
Leu Lys Glu Ser Gly Pro Gly Leu Val Ala Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser
PJ14 CTG AAG GAG TCA GGA CCT GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC ACA TGC ACC GTC TCA GGG TTC TCA
PCG1-1 ---
30 40 50
Leu Thr Gly Tyr Gly Val Asn Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu Gly Met Ile Trp Gly
PJ14 TTA ACC GGC TAT GGT GTA AAC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG GGA ATG ATA TGG GGT
PCG1-1 --- --- -T- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Ile
60 70
Asp Gly Ser Thr Asp Tyr Asn Ser Ala Leu Lys Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln Val
PJ14 GAT GGA AGC ACA GAC TAT AAT TCA GCT CTC AAA TCC AGA CTG AGC ATC AGC AAG GAC AAC TCC AAG AGC CAA GTT
PCG1-1 --- C-- --- --- ---
Gln
80 82a 82b 82c 90 95 100
Phe Leu Lys Met Asn Ser Leu Gln Thr Asp Asp Thr Ala Arg Tyr Tyr Cys Ala Arg D
PJ14 TTC TTA AAA ATG AAC AGT CTG CAA ACT GAT GAC ACA GCC AGG TAC TAC TGT GCC AGA G
PCG1-1 --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -CC TAC TAT AGA TAC GAC
Phe Ala Tyr Tyr Arg Tyr Asp
J3
PCG1-1 TGG TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA G
Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala

Figure 3) Hybridization of the PCG1-1 V_H probe to a Southern blot containing EcoRI digested genomic DNA. The V_H probe contains the V_H coding region from PCG1-1 rearranged to J3 (Fig. 1d). The arrow marks a 9.5 kbp V_H rearrangement in PCG1-1 DNA.

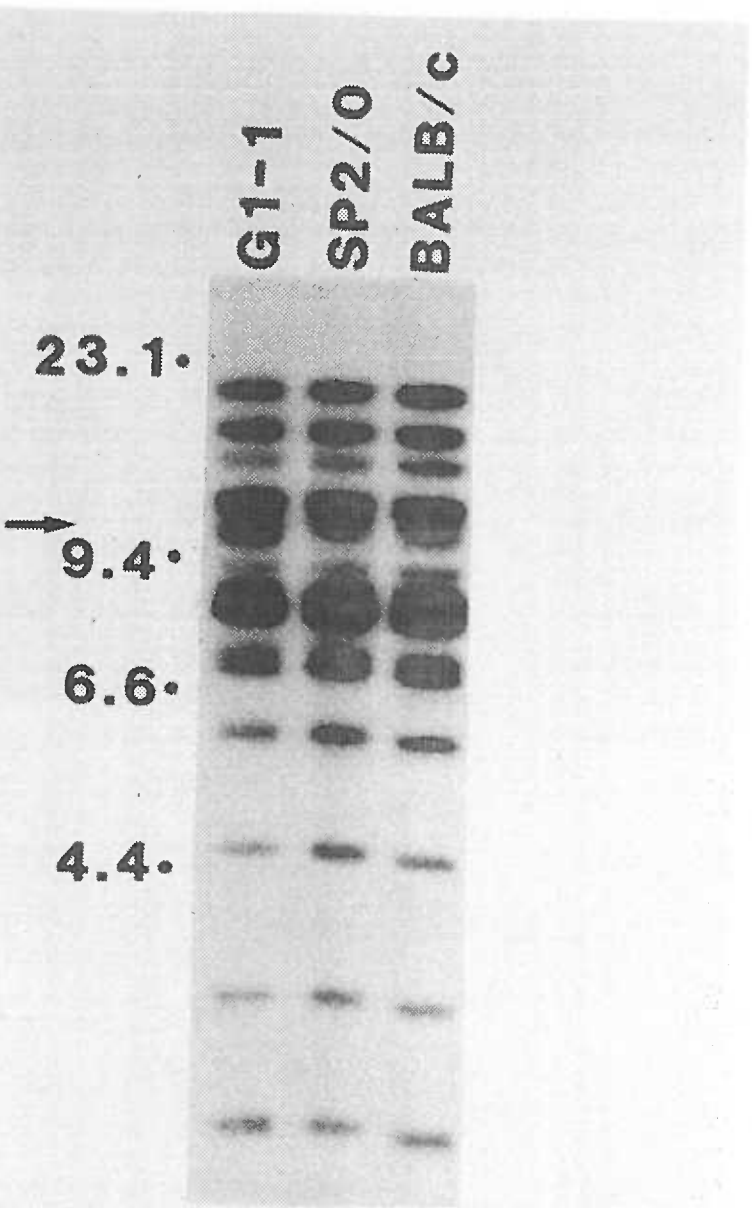

G1-1
SP2/O
BALB/c
23.1
9.4
6.6
4.4

Figure 4a) Hybridization of a J_H-specific probe to a Southern blot containing EcoRI digested genomic DNA from PCG1-1 and four λ_1-bearing Group II hybridomas.

Figure 4b) Rehybridization of the Southern blot shown in 4a to a 5' flanking region V_H probe. The 5' flanking probe contained 1.3 kbp of the upstream region from the cloned PCG1-1 V_H gene (Fig. 1d). The blot was hybridized to the 5' flanking probe following complete removal of the J_H probe used in the first hybridization. The arrows shown in A and B indicate a 9.5 kbp J_H and V_H rearrangement respectively.

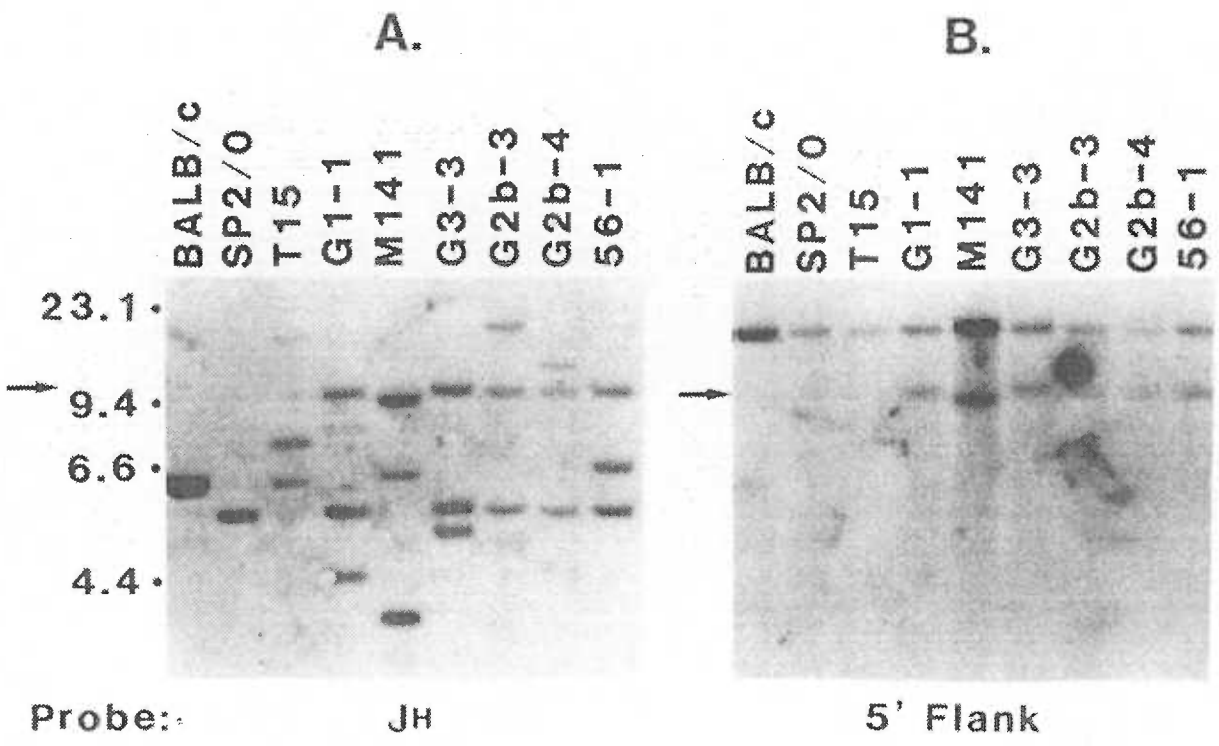

A.
B.
BALB/c
SP2/O
T15
G1-1
M141
G3-3
G2b-3
G2b-4
56-1
23.1
9.4
6.6
4.4
Probe:
JH
5' Flank

Figure 5a) Comparison of the VH amino acid sequences of the PC-binding myeloma M603 and the Group II PC-binding hybridoma PCG1-1.

Figure 5b) Comparison of D-J amino acid sequences of the PC-binding myelomas M603, T15, M511, and M167 with PCG1-1 and the I_{50} values for PC, NPPC and choline binding. I_{50} values taken from ref. 5.

a.

```
                                                       |---CDR1---|                         |--
                   10                                 30                                    50
M603     E V K L V E S G G G L V Q P G G S L R L S C A T S G F T F S D F Y M E W V R Q P P G K R L E W I A A

PCG1-1   Q - Q - K - - - P - - - A - S Q - - S I T - T V - - - S L T V Y G V N - I - - - - - - - G - - - L G M

         ----------------CDR2-----------------------|
          52 a b c                            70                    82 a b c          90
M603     S R N K G N K Y T T E Y S A S V K G R F I V S R D T S Q S I L Y L Q M N A L R A E D T A I Y Y

PCG1-1   I W[. . .]G D G S - D - N S A L - S - L S I - K - N - - - Q V F - K - - S - Q T D - - - R - -

               |---D---|-----------J-----------|
                    100 a b c
M603     C A R N Y Y G S T W Y F D V W G A G T T V T V S S

PCG1-1   - - - A - - R Y D - F A Y W G Q G T L V T V S A
```

b.

	|---D---||--J--|		I_{50}	
	95 100 a b c	PC	Choline	NPPC
PCG1-1	A Y Y R Y D W F A Y W	>10	>10	0.13
M603	N Y Y G S T W Y F D V	0.017	7.6	0.27
T15	D Y Y G S S Y W Y F D V	0.007	2.0	0.036
M511	D G D Y G S S Y W Y F D V	0.012	0.24	0.03
M167	D A D Y G D S Y F G Y F B V	0.20	0.89	0.053

Paper 3

Immunoglobulin variable region heptamer-nonamer signal sequence joined to rearranged D-J segment: Implications for the immunoglobulin recombinase mechanism

ABSTRACT

We have found a novel immunoglobulin gene rearrangement in a murine hybridoma in which a heavy chain variable region (V_H) heptamer-nonamer signal sequence is joined to the diversity segment (D) through head-to-head fusion. The heptamer-nonamer signal sequence and its adjacent 5' DNA are derived from the downstream flanking region of a germline V_H gene. Sequence analysis indicates that this adjacent DNA is homologous to the downstream flank of V_H108B and that it has characteristics of RNA processing which may suggest it was derived from an mRNA intermediate; these unusual features indicate that the segment is a processed gene. Because of head-to-head fusion the signal sequence and the flanking sequence are in opposite transcriptional polarity to D. The latter is joined correctly at its 3' border to a joining (J) gene segment. A γ1 constant region (but not μ) is located further downstream. Thus, this fragment has several features common to normal immunoglobulin heavy chain gene rearrangement despite the unusual joining event involving V-D.

Linkage of the V_H heptamer-nonamer signal sequence to D has not been observed previously. Although the signal sequence described is inverted with respect to D and J the endonucleolytic process that cleaved the signal sequence at the 5' border of the heptamer before rearranging it to D was accurate. We suggest that of the three functions associated with the recombinase reaction: recognition, cutting, and ligation, only

recognition and cutting may be limited to specific structures and that the ligation step may be less restricted since it is not confined to forming coding to coding or flank to flank joints. This aberrant ligation product suggests that the information leading to normal rearrangements may be found in structures that include more than the signal sequences or coding regions alone since the joining described here has spliced the incorrect end of a signal sequence to a coding region to yield a nonproductive recombination.

Formation of complete immunoglobulin (Ig) heavy (H) chain variable (V) regions requires two recombinational events involving V_H, diversity (D) and joining (J) segments (1-3). The rearrangement of these gene segments is brought about through the recognition of characteristic heptamer and nonamer signal sequences that adjoin the borders of V, D and J (1,4). The heptamer-nonamer sequences are separated by either 12 (D) or 23 (V and J) base pair (bp) spacers (1,4,5). Recombination occurs between a gene segment containing a 12 bp spacer and one containing a 23 bp spacer thus favoring joining between D and J_H and V_H and D (1,5). Products of IgH recombination include fused VDJ segments that no longer retain the signal sequence as well as D-J assemblies that have not involved V_H (6). Fused signal sequences have been detected from VJ joining in light (L) chain genes (7-9) but have not been reported from VD joining in H chain genes. Although they have not been observed previously, other products of a V_H to DJ joining (see Fig. 1) could include fusion of a V_H signal sequence to a D coding region and fusion of a V_H coding region to a D signal sequence. Here we describe a V_H recombination between a V_H signal sequence and a D coding region giving rise to a previously unrecognized product of the recombination reaction.

The recombinase enzymes for Ig remain undefined and the rules governing their activity are only partially understood. As pointed out recently, the orientation and relative positions of the individual genetic elements that must be recombined to form the coding sequences for the intact molecule are in most cases unknown as are the specific sequences that serve the joining process (10). Three activities have been attributed to the putative recombinase: 1) recognition of the heptamer-nonamer signal sequences via a DNA-binding activity that also

orients the DNA for recombination, 2) an endonuclease activity that cleaves at the borders of the signal sequence and, 3) a recombinase activity that ligates the cut ends of the coding regions after the signal sequence is removed (11). The recombinase reaction leading to the product described here appears to have functioned normally in steps 2 and 3 but failed in the orientation step so that it was the signal sequence rather than its upstream flanking region that was ligated to D. It is suggested that the proper orientation required to perform ligation may be a fourth activity, distinct from the signal sequence recognition step.

Materials and Methods

Construction and Screening of a Hybridoma DNA Library in Bacteriophage λEMBL4

DNA obtained from the anti-phosphocholine hybridoma, PCG1-1 (12) was partially digested with Eco RI and ligated into the bacteriophage vector λEMBL4 (13). Approximately $5x10^5$ phage recombinants were screened according to Maniatis et al. (14) for J_H containing sequences using a J_H-1-J_H-4 specific probe (15) after labeling by nick translation (16). J_H positive recombinants were re-screened for μ and γ_1 constant region-containing sequences using a γ_1 specific probe pM21γ1 prepared by Drs. J.M. Adams and S. Corey (17), and a μ specific probe, pRN12 provided by Dr. R. Near. The phage clone was characterized by limited restriction enzyme analysis using various enzymes as indicated in the text in accordance with manufacturer's guidelines (New England Biolabs, Beverly, MA, Bethesda Research Laboratories, Gaithersburg, MD, International Biologicals, Inc., New Haven, CT).

J_H Fragment Subcloning and DNA Sequence Analysis.

A 4.4 kbp Eco RI J_H fragment was subcloned into pBR325 (18) for restriction enzyme analysis. A 715 bp Hpa II/Bgl II fragment containing the J_H region was subcloned into M13 mp18/mp19 (19) and sequenced by the dideoxynucleotide chain termination method (20). DNA sequence comparisons were provided by Bionet™ National Computer Resources.

Southern Blot Analysis.

DNAs from the hybridoma PCG1-1, SP2/0 (fusion partner) and BALB/c liver were EcoRI digested (10u/μg) at 37°C for 6 hours. Approximately 5-10 μg of restricted DNAs were fractionated by gel electrophoresis (0.7%

agarose, tris acetate), blotted onto nitrocellulose and hybridized to ^{32}P-labeled probes as described previously (21). Filters were washed at 65°C (probe A) or 58°C (probe B) in .2XSSC, .1% SDS. Probe A is a 1.3 kbp Hpa II/Bgl II fragment that was obtained from the 4.4 kbp J_H fragment as shown in Fig. 2. Probe B is a 175 bp Rsa I/Rsa I insert cloned into M13 mp19 and was also obtained from the 4.4 kbp J_H fragment shown in Fig. 2. DNA probes were labeled with ^{32}P-dATP to a specific activity of 5-10 x 10^8 cpm/μg.

Results and Discussion

The anti-phosphocholine hybridoma PCG1-1 contains three J_H rearrangements detectable by Southern analysis of Eco RI digested DNA (12). The pattern in Fig. 2a indicates that the 4.4 kbp and 9.5 kbp bands are unique J_H rearrangements, whereas the 5.7 kbp band is also present in the myeloma fusion partner SP2/0. The 9.5 kbp fragment is a productive rearrangement encoding the H chain of an anti-phosphocholine IgG antibody whose sequence is unrelated to the rearranged sequences described here (Stenzel-Poore et al., manuscript in preparation). The 4.4 kbp fragment was isolated from a λ phage EMBL4 library containing PCG1-1 DNA partially digested with Eco RI (clone 2V-II). The 4.4 kbp J_H fragment was located on a 15.0 kbp fragment that also hybridized to a γ1 constant region probe; the J_H and γ1 hybridizing regions are separated by a 4.0 kbp Eco RI fragment (Fig. 2b). Thus, 2V-II consists of a rearranged J_H segment and a downstream γ1 constant region which may reflect a normal switch event from μ to γ1 since the clone did not hybridize to a μ specific probe (data not shown).

The 715 bp Bgl II/Hpa II DJ-containing fragment (Fig. 2c,d) shares identity with J_H-4. The sequence (Fig. 3a) which begins at the Hpa II site at position 715 and extends 5' consists of 97 bp identical to the 3' flank of germline J_H-4 followed 5' by the J_H-4 coding region (21). Homology with the germline J_H-4 sequence ends at position 573. The 17 bp segment immediately 5' of J_H is identical to the SP2.7 D segment (5). Thus, a D-J rearrangement in the correct translational reading frame is present. However, an inverted V_H recognition sequence lies immediately 5' of D. This unusual rearrangement consists of an inverted heptamer-23 bp spacer-nonamer signal sequence. This signal sequence and its flanking region are homologous to the 3' flanking region located immediately downstream of a known germline V_H gene, 108B (23,24) (Fig. 3b). The inverted sequence shows strong homology with the downstream flanking region of 108B. The signal sequence is separated from D by three bp that do not appear to have been derived from either the germline D coding region or the flanking region of V_H 108B (Fig. 3b, position 556). Such minisequences have been observed previously and are believed to represent nongermline encoded insertions of nucleotides to V_H or D before ligation (25). Homology with the flanking sequence of V_H108B extends from the heptamer recognition sequence downstream through the nonamer sequence and continues through a poly (A) addition signal AATAAA (26) at position 175. The close homology (76%) ends within the poly (A) tract where a gap of 10 bases in the germline V_H 108B sequence is required to maximize homology with 2V-II. This first 18 bp poly(A) sequence is separated by 21 bp from a second poly(A) segment of 26 bp. Hence, the hybridoma sequence and the 108B downstream flanking sequence diverge from one another at a point in 2V-II suggestive of RNA processing. These long poly(A) tracts close to

the putative poly(A) addition signal suggest the 2V-II sequence represents a processed V_H gene from an mRNA intermediate. While processed Ig constant region pseudogenes have been reported previously (27,28) processed V_H genes have not been found. A summary diagram of the structure of 2V-II as deduced from the nucleotide sequence is shown in Fig. 4.

It is possible that the processed segment exists in the germline as a complete V_H coding and flanking segment. If this were true it would provide additional support for transcritpion of unrearranged V_H genes; mRNA transcripts of such genes from pre B cells have contained the entire V_H coding region, the heptamer-nonamer recognition sequence as well as the poly(A) addition signal (29). Thus, this processed gene could have arisen through the transcription of an unrearranged V_H gene which was subsequently reverse transcribed and integrated into the genome although the site of integration is unpredictable. It is possible that in the hybridoma, PCG1-1, this processed element rearranged to D-J to produce the structure found on 2V-II (Fig. 4). Although this is a likely scenario it was not clear from the above data whether a complete V_H coding region was originally associated with the processed flanking region found in 2V-II. This could only be determined by cloning the germline equivalent of this processed segment. However, in order to consider cloning, it was necessary to determine whether the processed segment is indeed present in the germline or whether it arose during B-cell differentiation.

If the latter were correct the germline DNA would not be expected to contain the processed V_H segment. In order to distinguish between these possibilities we used two probes (Fig. 2c) one of which (probe A)

consisted of the adjacent 5' flank of the inverted V_H segment; the second contained the region homologous to 108B (probe B). If the processing of 2V-II occurred during B cell development probes A and B would not be expected to be adjacent in germline DNA as they were in the hybridoma and thus would hybridize to different fragments. If in contrast, the processed gene preexists in the germline, probes A and B would hybridize to a common fragment. A Southern blot of Eco RI digested PCG1-1 DNA, SP2/0 DNA (fusion partner) and BALB/c liver DNA (germline) hybridized to probe A is shown in Fig. 5a. Three bands (10.0, 5.8 and 3.0) are shared by liver, SP2/0 and PCG1-1 DNA. The unique 4.4 kbp band in PCG1-1 corresponds to the 4.4 kbp J_H rearrangement shown in Fig. 2a. Thus, the flanking sequence detected by probe A is present on three restriction fragments found in the germline and in a rearrangement to J_H in PCG1-1 (4.4 kbp). Since probe A was isolated from a fragment also containing the sequence homologous to the downstream flank of V_H 108B, probe A should also hybridize to Eco RI fragments containing the 108B flank. To determine which one of these fragment(s) might also contain the downstream segment of 108B, the region homologous to the downstream flank of 108B was cloned (Fig. 2c, probe B) and hybridized to Eco RI digested liver and hybridoma DNA. Probe B detects approximately 10 restriction fragments in liver, SP2/0 and PCG1-1 DNA (Fig. 5b) reflecting the multi-gene nature of the MPC11 V_H family of which 108B is a member (30). Importantly, however, probe B and probe A detect one germline band in common (10.0 kbp) indicating that the regions corresponding to A and B are associated in germline as well as in the hybridoma DNA. The observation that both A and B detected a 4.4 kbp band in PCG1-1 DNA (Fig. 5a,b) which also hybridized to J_H (Fig. 2a) but that no similar

rearrangement was detected in germline DNA serves as an internal control for the specificity of both probes. Thus, both the 5' flanking probe A and the probe for the 3' flank of 108B detect a common band in germline DNA suggesting that their association as detected in PCG1-1 also exists in the germline. Presumably the rearrangement of this DNA segment to D-J occurred during B cell development. Cloning and sequencing of the germline form of the processed gene will ultimately reveal whether it is associated with a V_H coding region and whether it has other features of RNA processing such as intron splicing and whether it is a functional V_H gene.

This is the first reported isolation of a V_H heptamer-nonamer signal sequence physically recombined to a D coding region and, thus, suggests that the signal sequence as well as the coding regions could serve as substrates for the putative recombinase in VDJ joining. Recombined signal sequences from L chain genes have been reported previously (7-9) and in one instance H chain DJ signal sequences have been joined inverted with respect to one another (25). Inversion has been suggested as the ordinary mechanism for functional V-J joining in L chains (31) and requires that the gene segments to be joined are oriented in opposite transcriptional polarity. The recombination product described here could result from an inversion of a V_H gene segment only if it were oriented in the germline in the same transcriptional polarity as D and J. Indirect evidence for the inversion model has been reported for V-J joining of the κ-chain locus (7,8); in vitro experiments using an expression vector support the feasibility of Vκ-gene inversion (31,32). More recently, direct evidence was obtained for an inverted T cell receptor Vβ gene located in the germline (33). The Vβ was inverted relative to the Dβ and

Jβ segments and was 3' of the Dβ, Jβ, and constant regions. Furthermore, in a T cell hybridoma that had undergone a rearrangement of the inverted Vβ gene both the appropriate VDJ coding joint and the reciprocal recombination product were linked on the same piece of DNA.

Sister chromatid exchange could also give rise to the recombination product described here and could yield an inverted V_H recognition sequence joined to the D region if the V_H gene segment were oriented in the germline in opposite transcriptional polarity to D-J. However, both models of rearrangement require that the V_H gene segment described here be oriented in the germline in opposite polarity to most V_H genes.

The recombination product described serves to define additional products that can result from recombination between V_H gene elements (Fig. 1). Although it is not obvious that the normal mechanisms of VDJ rearrangement were utilized we suggest this may have been the case. It was recently suggested that the signal sequence itself may be sufficient for recognition and recombination (11,34) and that the heptamer may provide the environment necessary for cleaving an appropriate sequence (11). The latter study reported a putative recombinase activity that was defined by the ability of nuclear extracts to cleave a particular dinucleotide pair $\frac{TG}{AC}$ near the heptameric signal sequence of Jλ1. It was suggested that since not all such dinucleotide pairs were cut, correct cleavage may require additional secondary or tertiary structural information which could be provided by the heptamer and/or the nonamer sequences (11,35). The endonucleolytic activity was apparently normal in the production of the recombinant that we describe since the J_H and D signal sequences appear to have been cleaved appropriately from their respective coding regions adjacent to the conserved heptamers (11,25);

what appears to have been aberrant is the ligation event which ordinarily links V_H to D, although the actual chemistry of the ligation also appears to have been normal. Thus, the real dysfunction may have been the orientation step in which one of the two segments (the heptamer-nonamer) was inappropriately placed in the alignment normally reserved for the V_H coding sequence. It is possible that the recombinase failed to displace the signal sequence prior to ligation. Although we have no evidence for this aspect it is possible that the secondary structure of the processed gene element (e.g. unusual poly (A) tracts) prevented the normal orientation process. Since the signal sequence itself is normal this could suggest that recognition for orientation involves a structure larger than the heptamer-nonamer. The putative recombinase itself including the ligation function could be impaired although this seems less likely as such enzymes are likely to be trans acting and must be functional in PCG1-1 since there is also a productive VDJ rearrangement.

This recombination product extends the range of possible products which could be obtained during Ig gene recombination, some of which may result in the nonproductive rearrangements common in B cells. Although our understanding of the number and structure of the genetic elements comprising Ig molecules is essentially complete, much remains to be learned regarding their regulation and assembly. As recently pointed out, joining of Ig genes serves as an important model for other DNA recombinational processes and elucidation of the enzymatic mechanisms associated with V gene joining will have broader biological significance (10). Defining the reactants, products and specificity of the recombination process will be essential to understanding the basic mechanisms of Ig rearrangement.

REFERENCES

1. Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D, and J_H. Cell 19:981.
2. Davis, M., K. Calame, P.W. Early, D.L. Livant, R. Joho, I.L. Weissman, and L. Hood. 1980. An immunoglobulin heavy-chain gene is formed by at least two recombinational events. Nature 283:733.
3. Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature 302:575.
4 Sakano, H., K. Huppi, G. Heinrich, and S. Tonegawa. 1979. Nature 280,288-294.
5. Kurosawa, Y., and S. Tonegawa. 1982. Organization, structure , and assembly of immunoglobulin heavy chain diversity DNA segments. J. Exp. Med. 155:201.
6. Early, P., C. Nottenburg, I. Weissman, and L. Hood. 1982. Immunoglobulin gene rearrangements in normal mouse B cells. Molec. and Cell Biol. 2:829.
7. Feddersen, R.M., and B.G. Van Ness. 1985. Double recombination of a single immunoglobulin κ-chain allele: Implication for the mechanism of rearrangement. Proc. Natl. Acad. Sci. USA 82:4793.
8. Lewis, S., N. Rosenberg, F.W. Alt, and D. Baltimore. 1982. Continuing kappa-gene rearrangement in a cell line transformed by Abelson murine leukemia virus. Cell 30:807.
9. Hochtl, J., C.R. Müller, and H.G. Zachau. 1982. Recombined flanks of the variable region and joining segments of immunoglobulin genes. Proc. Natl. Acad. Sci. USA 79:1383.

10. Baltimore, D. 1986. Inversion for gene construction. Nature 319:12.

11. Hope, T.J., R.J. Aguilera, M.E. Minie, and H. Sakano. 1986. Endonucleolytic activity that cleaves immunoglobulin recombination sequences. Science 231:1141.

12. Chang, S.P., R.M. Perlmutter, M. Brown, C.H. Heusser, L. Hood, and M.B. Rittenberg. 1984. Immunologic memory to phosphocholine IV. hybridomas representative of Group I (T15-like) and Group II (non-T15-like) antibodies utilize distinct V_H genes. J. Immunol. 132:1550.

13. Frischauf, A., H. Lehrach, A. Poustka, and N. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. 170:827.

14. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory) p. 320.

15. Calame, K., J. Rogers, P. Early, M. Davis, D. Livant, R. Wall, and L.Hood. 1980. Mouse Cμ heavy chain immunoglobulin gene segment contains three intervening sequences separating domains. Nature 284:452.

16. Rigby, P.W.J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237.

17. Adams, J.M., N.M. Gough, E.A. Webb, B.M. Tyler, J. Jackson, and S.Corey. 1980. Molecular cloning of mouse immunoglobulin heavy chain messenger ribonucleic acids coding for μ, α, γ_1, γ2a, and γ3 chains. Biochemistry 19:2711.

18. Prentki, P., F. Karch, S. Iida, and J. Meyer. 1981. The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. Gene 14:289.

19. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strain: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103.

20. Sanger, F., S. Nicklen, and A. Coulsen. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463.

21. Gough, N.M., and O. Bernard. 1981. Sequences of the joining region genes for immunoglobulin heavy chains and their role in generation of antibody diversity. Proc. Natl. Acad. Sci. USA 78:509.

22. Brown, M., M. Stenzel-Poore, M.B. Rittenberg. 1985. Immunologic memory to phosphocholine VII. Lack of T15 V_1 gene utilization in Xid anti-PC hybridomas. J. Immunol. 135:3558.

23. Givol, D., R. Zakut, K. Effron, G. Rechavi, D. Ram, and J.B. Cohen. 1981. Diversity of germline immunoglobulin V_H genes. Nature 292:426.

24. Cohen, J., K. Effron, G. Rechavi, G., B. Yinon, R. Zakut, and D. Givol. 1982. Simple DNA sequences in homologous flanking regions near immunoglobulin V_H genes: A role in gene interaction? Nucl. Acid Res. 10:3353.

25. Alt, F.W., and D. Baltimore. 1982. Joining of immunoglobulin heavy chain gene segments: implication from a chromosome with evidence of three D-J_H fusions. Proc. Natl. Acad. Sci. USA 79:4118.

26. Nevins, J.R. 1983. The pathway of eukaryotic mRNA formation. Ann. Rev. Biochem. 52:441.

27. Hollis, G.F., P.A. Hieter, W.O. McBride, D. Swan, and P. Leder. 1982. Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA-type processing. Nature 296:32.

28. Battey, J., E.E. Max, W.O. McBride, D. Swan, and P. Leder. 1982. A processed human immunoglobulin E gene has moved to chromosome 9. Proc. Natl. Acad. Sci. USA 79:5956.

29. Yancopoulos, G.D., and F.W. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. Cell 40:271.

30. Bothwell, A.L.M., M. Paskind, M. Reth., T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1981. Somatic variants of murine immunoglobulin λ light chains. Cell 24:625.

31. Lewis, S., A. Gifford, and D. Baltimore. 1985. DNA elements are asymmetrically joined during the site-specific recombination of kappa immunoglobulin genes. Science 228:677.

32. Lewis, S., A. Gifford, and D. Baltimore. 1984. Joining V_K to J_K gene segments in a retroviral vector introduced into lymphoid genes. Nature 308:425.

33. Malissen, M., C. McCoy, D. Blanc, J. Trucy, C. Devaux, A.M. Schmitt-Verhulst, F. Fitch, L. Hood, and B. Malissen. 1986. Direct evidence for chromosomal inversion during T-cell receptor β-gene rearrangements. Nature 319:28.

34. Yancopoulos, G.D., T.K. Blackwell, H. Suh, L. Hood, and F.W. Alt. 1986. Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. Cell 44:251.

35. Desiderio, D., and D. Baltimore. 1984. Double-stranded cleavage by cell extracts near recombinational signal sequences of immunoglobulin genes. Nature 308:860.

ACKNOWLEDGEMENTS

We thank P. Stenzel, M. Brown, C. Faust and R. Bestwick for helpful discussion and advice and U. Bruderer, A. Buenafe, J. Hartmann, F. Makowski, N. Keller, and T.V. Tittle for critical review of the manuscript. Computer resources were provided by Bionet ™ National Computer Resources for Molecular Biology which is supported by Grant 1U41-RR-01685-02 from the National Institutes of Health.

Fig. 1 Model defining the reactants and products that could result from recombination between V_H gene elements bearing heptamer-23 bp spacer-nonamer sequences and D elements containing heptamer-12 bp spacer-nonamer sequences. Intermediate formation may involve sequence specific strand scission adjacent to the heptamer. Ligation of the spliced elements results in formation of fused heptamer-nonamer sequences each derived from the initial V_H and D elements (product I) and the frequently observed V_H-D productive rearrangement (product II). Additional products proposed include joining of a V_H heptamer-nonamer sequence to a D element (product IV) and a possible product (not observed), i.e. V_H coding region joined to a D heptamer-nonamer sequence (product III).

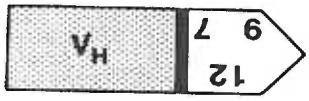
Initial Reactants

Intermediates

Products

I. Possible

II. Known



III. Possible

IV. Observed Here

Fig. 2a) Southern blot analysis of PCG1-1 hybridoma DNA detects three J_H rearrangements. DNAs from PCG1-1, SP2 (fusion partner) and BALB/c liver were Eco RI digested, gel electrophoresed, transferred to nitrocellulose and hybridized to a ^{32}P-labelled J_H specific probe. Size markers are indicated in kb.

b) λ phage clone 2V-II was obtained from a library containing PCG1-1 DNA partially digested with Eco RI and cloned into the λ bacteriophage vector EMBL-4 by screening with a J_H probe. The λ clone contains a 4.4 kbp J_H fragment and a downstream 6.2 kbp γ_1 fragment separated by a 4.0 kbp fragment. Eco RI digestion also yielded a 0.4 kbp fragment which was not mapped. The γ_1 fragment was detected using a γ_1 specific probe.

c) Limited restriction map of the 4.4 kbp Eco RI fragment containing the J_H region cloned into the plasmid vector pBR325. Restriction sites are indicated by the vertical lines and abbreviations are as follows: E, Eco R1; P, Pst I; H, Hpa II; B, Bgl II; X, Xba I. Probes A and B shown directly below the restriction map represent probes used to determine the presence of homologous sequences in germline DNA (Fig. 5).

d) Sequencing strategy of a 715 bp Bgl II/Hpa II fragment containing the J_H region. The fragments obtained from this DNA segment were subcloned into M13 mp18/19 and sequenced by the dideoxynucleotide chain termination method. R = Rsa I.

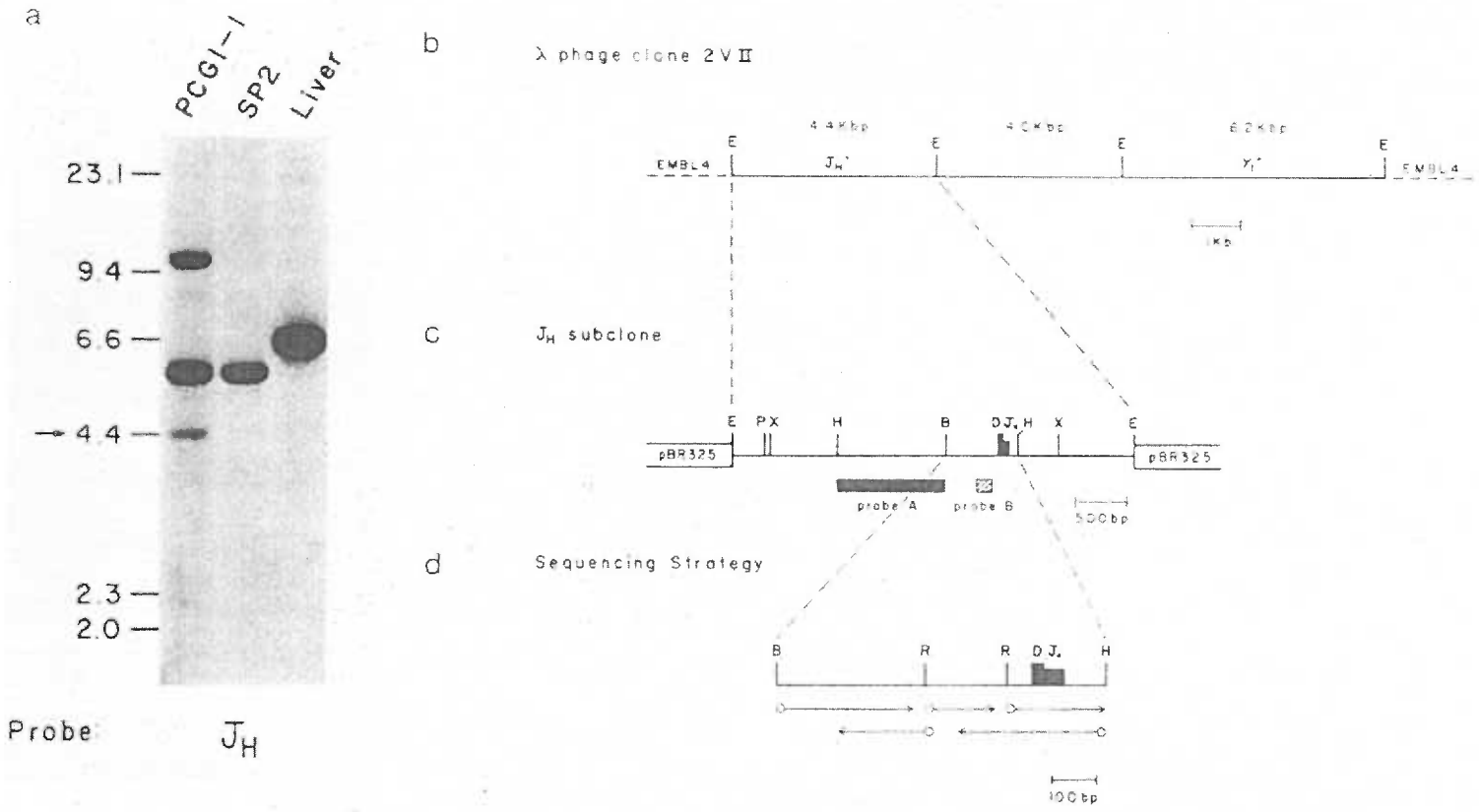
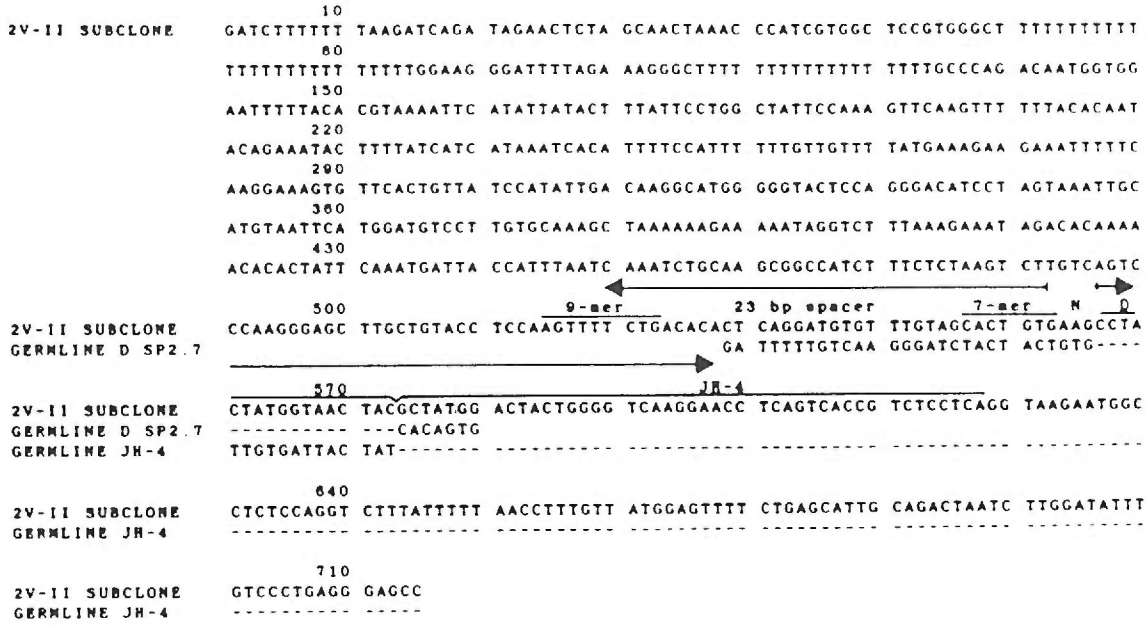

a
PCG1-1
SP2
Liver
23.1
9.4
6.6
4.4
2.3
2.0
Probe
J_H
b
λ phage clone 2VII
4.4Kbp
4.0Kbp
6.2Kbp
E
EMBL4
J_H'
V_1'
1Kb
c
J_H subclone
E
PX
H
B
DJ$_H$
X
pBR325
probe A
probe B
500bp
d
Sequencing Strategy
B
R
DJ$_H$
100bp

Fig. 3 Nucleotide sequence of an inverted V_H-flanking segment joined to D-J.

a) Sequence comparison of the 2V-II subclone to germline D (SP2.7) and J_H-4 regions.

b) Inverted complement of the 2V-II subclone nucleotide sequence shown extending 5' of the rearranged D-J joint in a. Sequence comparison of the 2V-II subclone to the downstream flanking region of the germline V_H gene-108B. Note that the 23 bp spacer found adjacent to D is characteristic of a V_H gene spacer rather than the 12 bp spacer typically found adjacent to D segments. The sequence was obtained by the dideoxy chain termination method. Nucleotide identities with the upper lines are shown with dashes. The sequence comparisons were provided by Bionet™ National Computer Resources. The arrows indicate 5' to 3' transcriptional orientation. Asterisks indicate gaps introduced to maximize DNA homology. Open regions in the 108B sequence indicate deletions as originally reported (24).

a.


2V-II SUBCLONE
GERMLINE D SP2.7
GERMLINE JH-4
9-mer
23 bp spacer
7-mer
N
D
JH-4

b.

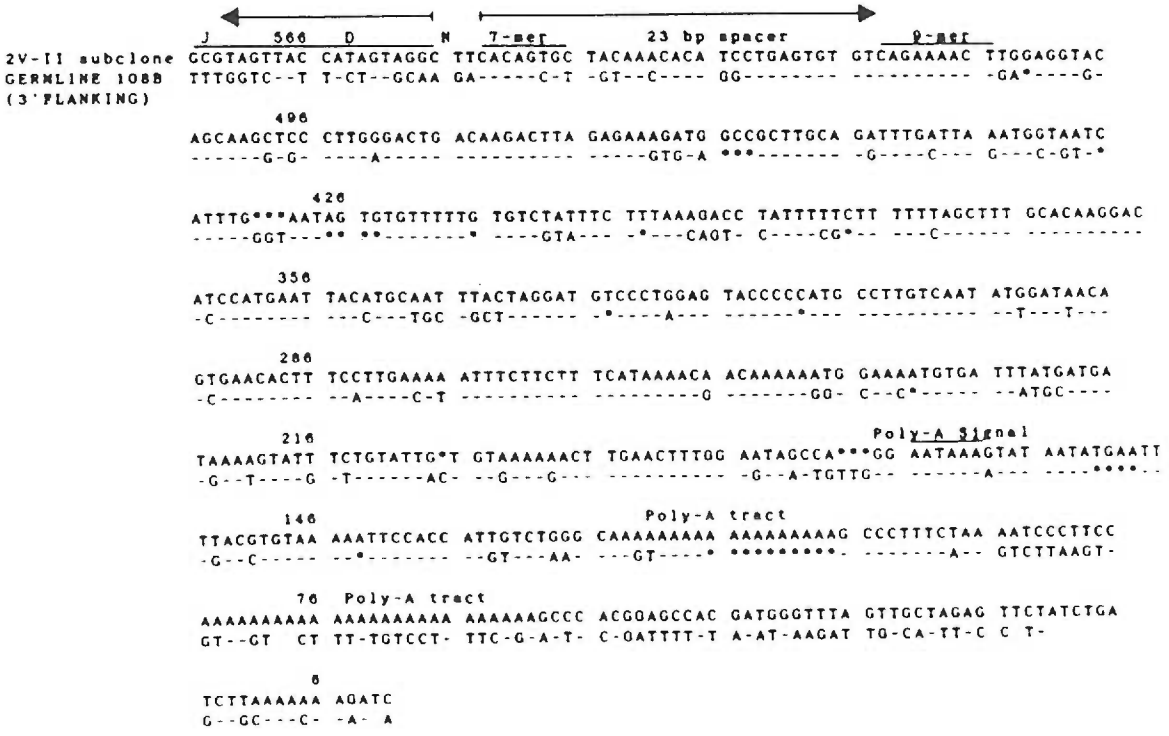

J
D
N
7-mer
23 bp spacer
9-mer
2V-II subclone
GERMLINE 108B
(3'FLANKING)
Poly-A Signal
Poly-A tract
Poly-A tract

Fig. 4 Schematic representation of the structure of 2V-II as deduced from the nucleotide sequences in Fig. 3. A rearranged D-J segment may have joined directly to the V_H heptamer-nonamer signal sequence and 3' flank of the processed V_H gene--V_H108B. The heptamer-nonamer sequence and downstream flank of V_H108B are inverted with respect to D-J resulting in a head-to-head fusion of the gene elements. The wavy arrows indicate direction of transcription.

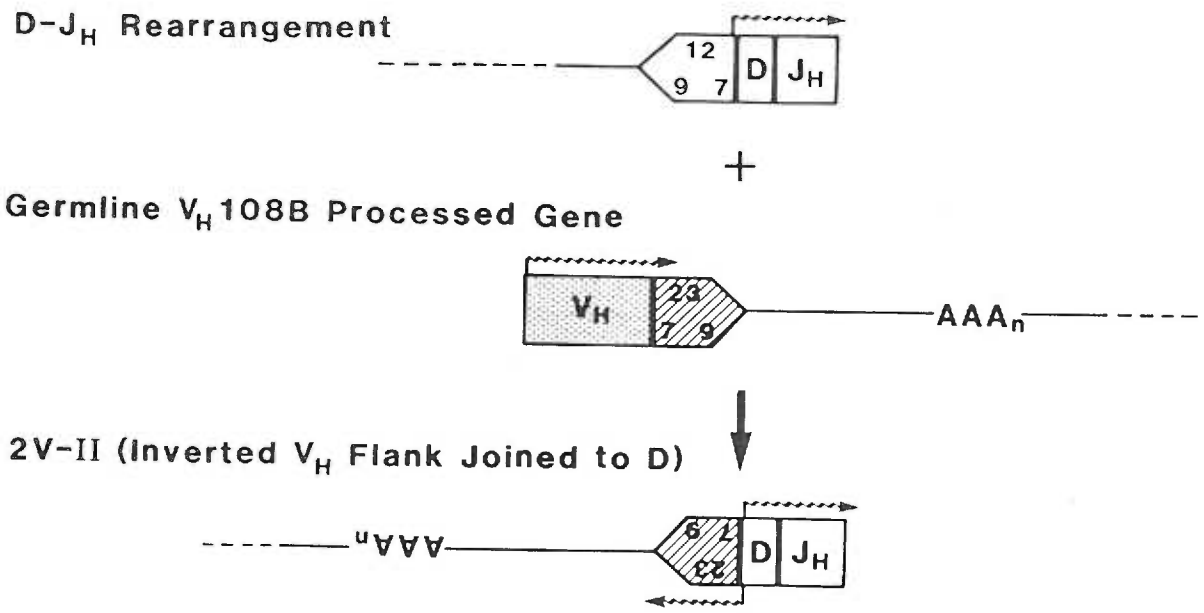
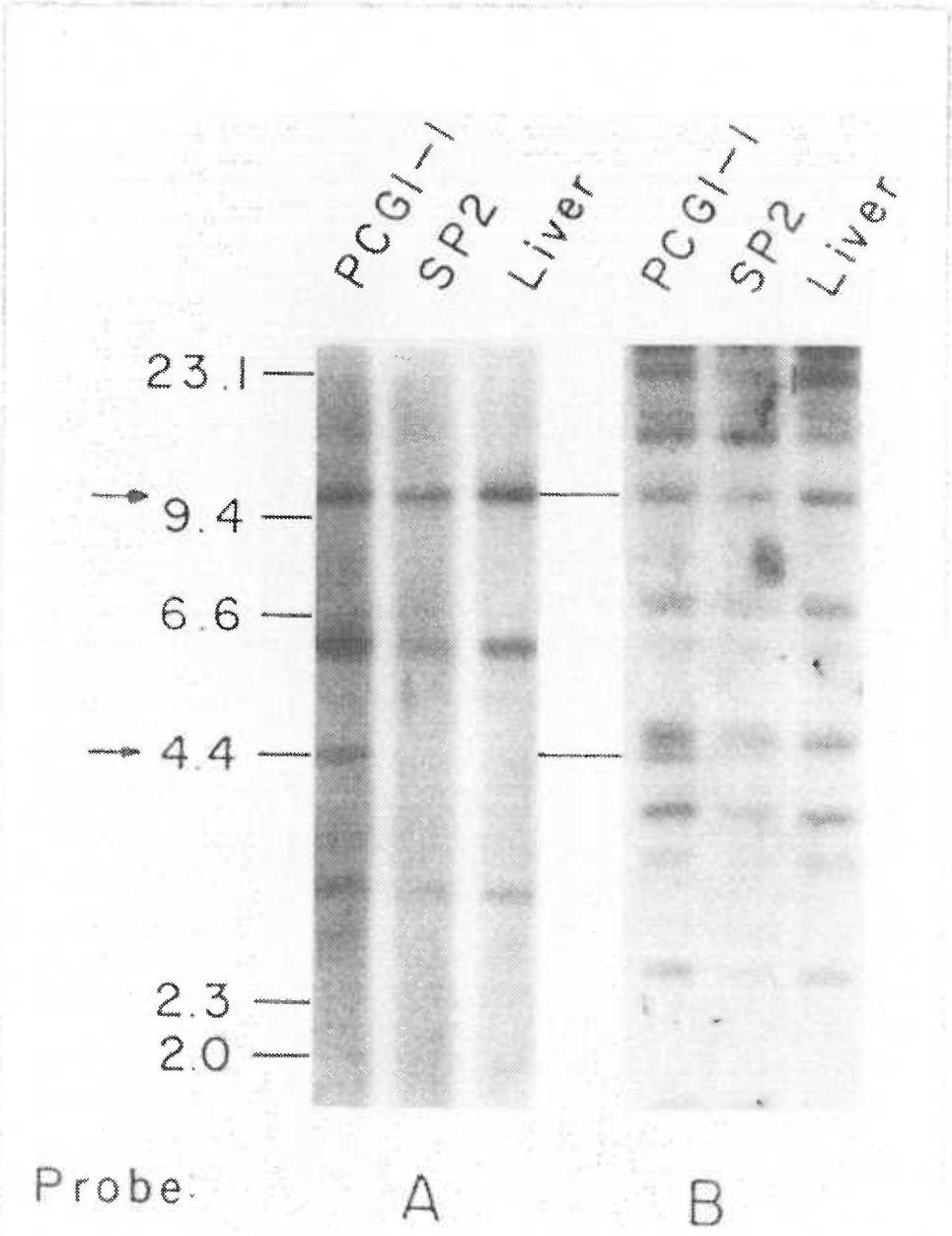

D-J_H Rearrangement
12
9
7
D
J_H
+
Germline V_H108B Processed Gene
V_H
23
7
9
AAA_n
2V-II (Inverted V_H Flank Joined to D)
AAA_n
6
7
22
D
J_H

Fig. 5 Southern blot analysis reveals the presence of the processed V_H 2V-II segment in the germline. PCG1-1 (hybridoma), SP2 (fusion partner) and liver (germline) DNAs were treated as described in Fig. 1 and hybridized with a, probe A; and b, probe B. Probes A and B were derived from the subclone 2V-II and contain DNA fragments located 5' of the D-J joint (Fig. 1c). Probe A detects sequences located next to the region found to be homologous with the downstream flanking region of V_H-108B. Probe B detects fragments homologous with the downstream flanking region of the germline V_H108B gene but does not extend through the poly(A) tract. The arrows indicate a common rearranged fragment (4.4 kb) detected by probe A and probe B and a common germline band at 10.0 kbp.


PCG1-1
SP2
Liver
PCG1-1
SP2
Liver
23.1
9.4
6.6
4.4
2.3
2.0
Probe:
A
B

APPENDIX

The following appendix contains experiments that further address the origins of V region diversity in the memory antibody response to PC. The experiments extend the findings presented in Papers 1 and 2 and are presented here in support of the hypothesis that additional phenotypic diversity can be obtained through the expression of:

1) V_H and V_L genes not observed in the primary response and,
2) V genes derived from elements observed in the primary reponse, but in new V_H-V_L combinations.

A discussion of these findings appears in the Summary and Discussion section of this thesis.

INTRODUCTION

The use of lambda light chains (λ) in the anti-PC response is rare and thus far only three such hybridomas have been reported (1,2); one of which is a Group II antibody expressing λ_2 that we have previously described (2) and two obtained from outbred wild mice (1). We have isolated an additional eight anti-PC hybridomas that express λ from BALB/c mice undergoing a memory response and are of the Group II binding phenotype and IgG isotype (3). Seven of the eight monoclonals have been found to use λ_1 and one uses λ_3.

In order to determine whether these antibodies utilize a single or limited set of V_H genes Southern and Northern blot analyses were performed. Restriction digests of hybridoma DNA probed with a J_H-specific probe (Appendix Figure 1) indicate that four of the hybridomas share a common rearrangement suggesting that they express the same V_H gene rearranged to the same J_H segment. We have identified the V_H gene of these four hybridomas as V_H M141 (which is a member of the Q52 family) (4) based on the finding that all share a common V_H rearrangement using a 5' V_H flanking probe that has been shown to be specific for the M141 V_H gene (Paper 2). Interestingly, the same V_H gene is found in association with V_k1-3 in a prototype Group II hybridoma, PCG1-1. The remaining four λ-bearing hybridomas exhibit a diverse pattern of J_H rearrangements indicating that there is heterogenous V_H usage among them. Northern blot analysis of the hybridomas indicates that two are using a V_H gene related to V_H M141, a Q52 family member. Thus, the Q52 V_H family contributes to all but two of the eight λ-bearing hybridomas described here. The finding that Q52 gene-family members have been found

associated with two different light chains (V_k1-3 and λ_1) in Group II antibodies may indicate that sequences common to members of this V_H family are particularly well suited to generating Group II-like combining sites in association with a particular set of V_L genes (since only V_k1-3 and λ_1 have been found thus far, the light chain set may be small, however until a larger number of Group II hybridomas have been examined the size of the set is uncertain).

Although preferred associations of V_λ with a limited set of V_H genes may govern the overall diversity in λ anti-PC antibodies, the use of λ in these Group II antibodies represents a further degree of heterogeneity generated in the anti-PC memory response.

It has been proposed that additional diversity in the late stages of an immune response may be the product of combinational diffusion (5,6) i.e., recruitment of clones that express V gene elements found in the primary response in combination with new genes not observed in the early stages. Since the major V gene element expressed in the primary anti-PC response is the product of the V_H1 gene it was of interest to determine whether this gene or related family members (S107) could give rise to Group II antibodies found in the memory response and thus contribute to the phenotypic heterogeneity observed in the secondary response. Northern blot analysis of RNA from a collection of Group II hybridomas indicates that the S107 family as well as the Q52 family contribute to the increased diversity observed in the memory response. Moreover, two hybridomas have been identified that express the V_H1 gene product in combination with V_k1-3 which provides further support for additional diversity arising through combinational diffusion.

METHODS

Southern blot analysis. High molecular weight DNA was prepared by the guanidinium thiocyanate/cesium chloride method (7) or by the method of Blin and Stafford (8). Ten μg of EcoRI digested DNA was electrophoresed (0.7% agarose) for 17 hr and transferred to nitrocellulose according to Southern (9). Prehybridization and hybridizations were performed at 42°C as described previously (10) and in Paper 2. The J_H specific probe (11) was labeled to a specific activity of 5.0×10^8 dpm/μg using random oligonucleotide primer extension (12) and hybridized at 7.0×10^7 dpm/filter. The filter was washed three times at low stringency in 2X SSC (1X SSC = 0.15M sodium chloride, 0.015M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at room temperature and three times at high stringency in 0.2X SSC, 0.1% SDS at 55°C. A 3.2 kbp probe containing all four BALB/c J_H genes cloned into pBR322 (11) was kindly provided by Drs. R. Perlmutter and L. Hood.

Northern blot analysis. Total RNA was isolated from tissue culture cells or tumors using the guanidinium thiocyanate/cesium chloride method (7). 15 μg of total or 0.5 μg of poly (A^+) RNA was fractionated by formaldehyde-agarose electrophoresis (2.2M formaldehyde, 1.0% agarose) for 18 hr (20 volts), rinsed twice in water, and soaked in 20 X SSC prior to blotting onto nylon membranes (Biotrans, ICN). The RNA was allowed to transfer for at least four hr. The nylon membranes were baked at 80°C for one hr, prehybridized, hybridized, and washed at 55°C (J_H probe) or 65°C (V_H probes M141 and S107) exactly as described (Paper 2). DNA probes were radiolabeled (^{32}P dATP) by random primer extension (12) to a

specific activity of 3.0-6.0 x 10^8 dpm/μg and membranes were hybridized at 3.-6.0 x 10^7 dpm/membrane. A Q52 V_H family probe containing the V_H coding region corresponding to the M141 gene (Paper 2) was used to determine if any hybridomas utilized a Q52 family gene. A cDNA probe containing the V_H coding region of the S107 myeloma (13) was used to detect S107 V_H gene family usage. To remove the probe completely prior to rehybridization, the membranes were washed at 65°C for 60 min in 10 mM phosphate (pH 6.5) and 50% formamide followed by one wash at room temperature for 15 min in 2X SSC.

APPENDIX TABLE I

PROPERTIES OF λ - BEARING GROUP II ANTI-PC HYBRIDOMAS[a]

Hybridoma	Isotype H	Isotype L	Strain	I_{50} (mM) PC	I_{50} (mM) NPPC	M141 Gene[b]	Q52 Family[b]
PCG2b-3	γ2b,	λ1	BALB/c	>10	0.53	+	+
PCG2b-4	γ2b,	λ1	BALB/c	>10	0.67	+	+
PCG3-3	γ3,	λ1	BALB/c	>10	0.44	+	+
aPC1-56-1	γ1,	λ1	BALB/c	>10	0.063	+	+
PCG1-10	γ1,	λ1	BALB/c	>10	0.08	-	+
PCG1-11	γ1,	λ1	BALB/c	>10	0.28	-	(+)[d]
PCG1-12	γ1,	λ1	BALB/c	>10	0.087	-	-
PCG1-9	γ1,	λ3	CBA/N	>10	0.8	-	-
PCG3-2	γ3,	λ2	F_1	>10	0.007	-	-

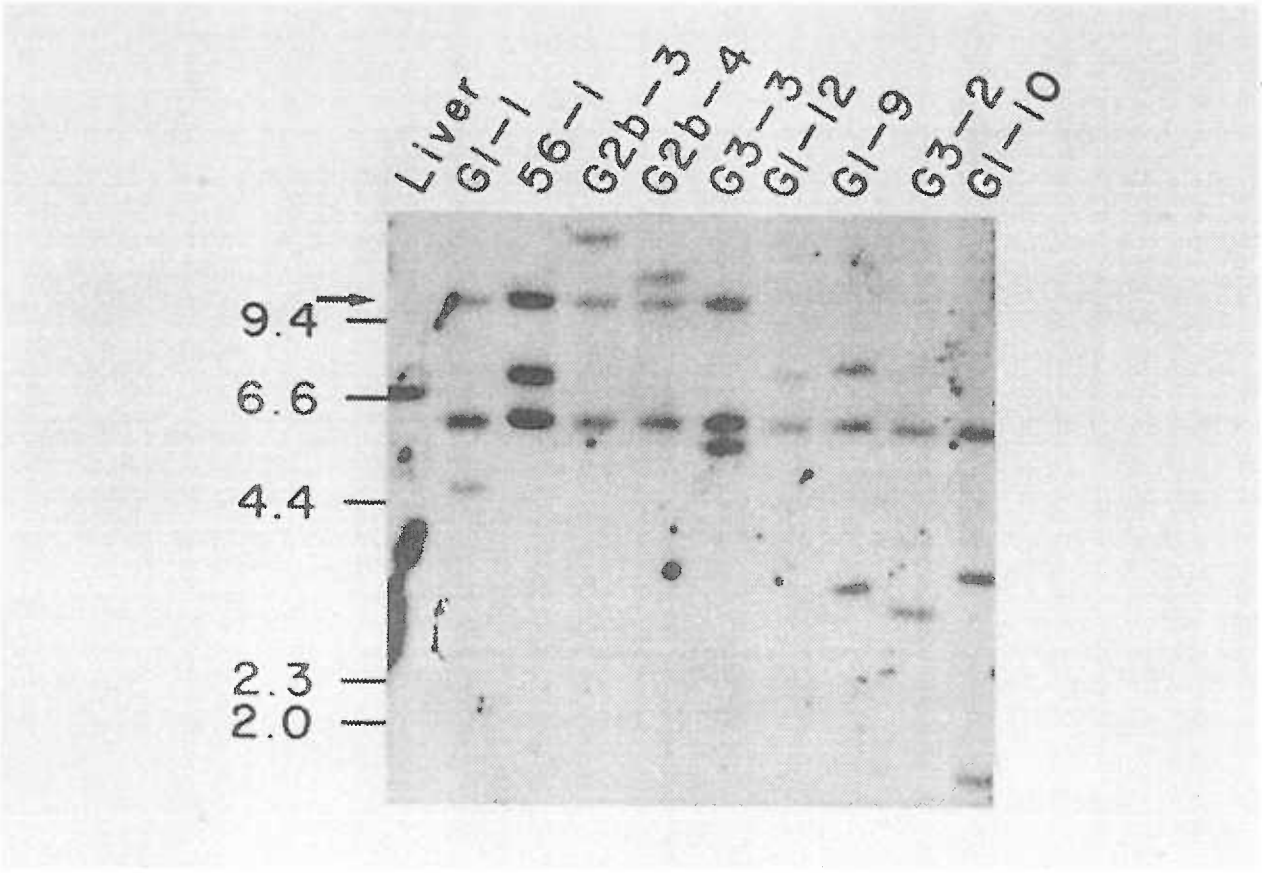
[a]Hybridoma generation and characterization described in detail in ref. (3). PCG3-2 has been described previously (2).

[b]V_H gene identification made by Southern blot hybridization using an M141 specific 5' flanking probe (described in Paper 2). + indicates M141 gene rearrangement, - indicates germline configuration as detected by the 5' flanking probe.

[c]Northern blot hybridization results; + and - indicate a positive or negative signal respectively, using a Q52 V_H family probe.

[d]Northern blot analysis was not done. PCG1-11 and PCG1-10 share both productive and non-productive J_H rearrangements (data not shown) indicating that they are likely to be clonally related and express the same V_H gene (Q52 family member); therefore PCG1-11 is inferred to be Q52 positive.

Appendix Figure 1. J_H rearrangements of λ-bearing Group II hybridomas. DNAs were EcoRI digested, blotted and hybridized to a probe containing all four J_H gene segments. DNA source is indicated at the top of each lane. The arrow indicates a common 9.5 kilobase pair (kbp) shared between the κ hybridoma PCG1-1 (Group II) and several λ hybridomas. Molecular weight markers are in kbp.


Liver
G1-1
56-1
G2b-3
G2b-4
G3-3
G1-12
G1-9
G3-2
G1-10
9.4
6.6
4.4
2.3
2.0

APPENDIX TABLE II

V_H GENE EXPRESSION IN ANTI-PC HYBRIDOMAS DERIVED FROM THE MEMORY POOL

Hybridoma	Isotype	Group	Strain	V_L[a]	V_H Family[b]
PCG1-1	γ1,κ	II	BALB/c	κ1-3	Q52 (M141)
aPC-12-3	ε, κ	II	BALB/c	κ1-3	Q52
aPC-104	γ3,κ	II	BALB/c	κ1-3	S107(V_H1)
aPC-111-1	μ, κ	II	BALB/c	κ1-3	S107(V_H1)
PCG2a-1	γ2a,κ	II	F_1[c]	κ1-3	S107
aPC-1-52	γ1,κ	II	BALB/c	κ1-3	other
PCG2b-2	γ2b,κ	II	F_1	κ8	other
PCG2b-1	γ2b,κ	III[d]	F_1	κ24	other
PCG1-2	γ1,κ	III[d]	BALB/c	κ24	other
PCM-24	μ, κ	I	F_1	κ24	S107
PCM-13	μ, λ2	I	BALB.B	λ2	S107

[a] V_L gene assignments were made by isoelectric focusing analysis or NH_2 terminal amino acid sequencing and are taken from ref. (2,14) or unpublished data.

[b] V_H family assignments were made by northern blot hybridizations using S107 or Q52 family probes. Parenthesis indicates the specific member of the family used and was determined by Southern blot analysis.

[c] (BALB/c X CBA/N) F_1 male mice.

[d] Binds specifically to PC-protein, but has no measurable affinity for free PC or NPPC.

REFERENCES

1. Cancro, M.P. and D. Hilbert. 1984. Characterization of phosphorycholine-binding antibodies from low responder (CNV) mice. UCLA Sym. Mol. Cell Biol. 18:467-472.

2. Todd, I., M. Brown and M.B. Rittenberg. 1985. Immunologic memory to phosphorycholine. VI. Heterogeneity in light chain gene expression. Eur. J. Immunol. 15:177-183.

3. Rittenberg, M.B., T.J. Hall and M. Stenzel-Poore. 1986. Immunologic memory to phosphocholine. IX. Utilization of lambda light chains in the memory antibody response to PC-KLH. in preparation.

4. Brodeur, P.H. and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse. I. One hundred Igh-V genes comprise seven families of homologous genes. Eur. J. Immunol. 14:922-930.

5. Manser, T., L.J. Wysocki, T. Gridley, R.I. Near and M.L. Gefter. 1985. The molecular evolution of the immune response. Immunol. Today 6:95-101.

6. Berek, C., G.M. Griffiths and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. Nature 316:412-418.

7. Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory) p. 196.

8. Blin, N. and D.W. Stafford. 1976. A general method for the isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. 3:2303.

9. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

10. Brown, M., M. Stenzel-Poore and M.B. Rittenberg. 1985. Immunologic memory to phosphocholine. VII. Lack of T15 V1 gene utilization in Xid anti-PC hybridomas. J. Immunol. 135:3558-3563.

11. Calame, K., J. Rogers, P. Early, M. Davis, D. Livant, R. Wall and L. Hood. 1980. Mouse C_{μ} heavy chain immunoglobulin gene segment contains three intervening sequences separating domains. Nature 284:452-455.

12. Feinberg, A. and B. Vogelstein. 1982. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analyt. Biochem. 132:6-13.

13. Early, P., H. Huang, M. Davis, K. Kalame and L. Hood. 1980. An immunoglobulin heavy chain variable region is generated from three segments of DNA: V_H, D and J_H. Cell 19:981-992.

SUMMARY AND DISCUSSION

Summary of Principal Findings.

The antibody response to phosphocholine is considerably more complex than was originally proposed (1-3). In BALB/c mice, the primary phase is restricted; the T15 idiotype dominates and the major antibody-binding phenotype is Group I (significant affinity for PC and NPPC). In sharp contrast to the homogeneous character of the primary response, the memory response is comprised of Group I and Group II antibodies (significant affinity for NPPC, bind PC weakly) which dominate distinct isotypes and express different idiotypes (4,5). Although Group II antibodies have not been demonstrated in the early primary response, it is likely that B cells giving rise to these antibodies exist at the time of initial immunization but remain undetected due to relatively low precursor frequency and/or display different triggering requirements such that they only become apparent during the later stages of the response.

Through extensive sequence analysis, a great deal of information has been acquired regarding the genetic composition of Group I antibodies (6,7). These studies demonstrate that the phenotypic restriction observed in Group I antibodies reflects a similar state of genetic restriction that results from use of a single V_H-J_H combination (V_H1-J_H1) in association with only three V_L gene products (V_k22, V_k24 and V_k8) rearranged to J_k5. Based on these findings it was of obvious interest to determine whether Group II antibodies evolved through the use of genes also used by Group I antibodies (perhaps in altered context or in mutated

form) or whether additional gene elements were responsible for the expression of this new binding phenotype.

The goal of this thesis project was to determine the molecular basis for Group II antibodies generated in the memory response. The hypothesis tested was that the heterogeneity observed in the anti-PC memory response originates from recruitment of clones expressing additional V genes not previously observed in the primary repertoire rather than from selection of somatic variants that express Group I-like genes but exhibit Group II binding characteristics. The principal findings of this research are:

1) The V_H identity of a prototype Group II antibody has been determined by molecular cloning; nucleotide sequence analysis was performed which indicates that the gene is derived from the M141 V_H element, a gene novel in the anti-PC response.

2) The M141 V_H gene was found in combination with two distinct light chain genes (V_k1-3 and V_λ1) in Group II antibodies; both of the V_L genes represent genes that have not been observed in the primary response to PC and thus support the notion that diversity in the later stages of this response can result from the expression of genes not found in the primary response.

3) The genetic basis for the lack of T15 idiotype expression in anti-PC antibodies produced in immune defective (Xid) mice was shown to be due to the lack of utilization of the V_H1 gene which is responsible in part for the formation of the $T15^+$ idiotype, thus the Xid produces an exceptionally high level of Group II antibodies which was shown previously by others (8,9).

4) The V_H1 gene product of Group I antibodies combined with Group II light chains can generate the Group II antibody phenotype.

In addition, at least one other member of the S107 family was found in a Group II antibody indicating that members of this V_H family contain the necessary V_H sequence information to allow the formation of a Group II-like binding site.

Thus, the experimental hypothesis turned out to be essentially but not entirely correct; the Group II binding phenotype displays striking genetic complexity compared to Group I antibodies resulting from use of a variety of V_H and V_L combinations; some of the new combinations evolve from entirely new gene entities not previously observed in primary antibodies, while others result from V genes (V_H or V_L) expressed in the primary response in association with new V_H or V_L genes such as V_H1 from primary antibodies combined with V_k1-3. It is now clear that the majority of the Group II response originates as new genotypes. No Group II antibodies were detected that could be ascribed solely to the genes of the T15 clonotype; although as indicated above some of these genes were used in combination with new genes. The extent of somatic mutation in T15 V genes found in new V_L or V_H combination is not yet clear. It remains to be seen whether future studies will reveal Group II antibodies derived solely through somatic mutation of the T15 clonotype.

Discussion of Findings and Future Endeavors.

The various H and L chain combinations that have been observed in Group I and Group II antibodies (hybridomas) are shown in Figure 1. The H and L chains shown along the x and y axis respectively contribute to the various combinations observed in memory-pool-derived Group I (●) and Group II (■) antibodies. Novel combinations comprised of V genes

observed in the initial phase of the response in association with new H or L chains expressed only in the memory response are indicated by the hatched symbols (⊘,⊠). These data demonstrate that maturation of the PC response culminates in extensive genetic diversity. This pattern of immune development differs sharply from the anti-ARS response which initiates heterogeneously and evolves into a highly focused response dominated by a single idiotype (10,11). The basis for selection of a single clonotype from a pool of ARS-binding B cells has been proposed to lie in the intrinsically high affinity receptor encoded by the Id^{CRI} V_H and V_L germline genes. The development of diversity from an initally contracted antibody response such as that seen in response to PC-KLH has been observed in the anti-oxazolone (OX) system (12,13) and the sources of diversity appear to be similar in the two antigen systems. However, an important aspect regarding the evolution of Group II antibodies remains obscure; that is, the fact that the primary anti-PC response appears to be devoid of Group II antibodies. The OX response was also found to be missing those clonotypes in early stages that later were well represented in the memory response. However, recent evidence provided by Kaartinen et al. suggests that only 30-50% of the response is actually due to the clonotype V_HOx1-V_LOx1 previously reported to represent ~ 75% of all hybridomas characterized from a primary anti-OX response (14). The early response clearly contains some of the clonotypes observed in the late response. Moreover, these clones were found generally to exhibit lower affinities for OX than V_HOx1-V_LOx1 which may offer an explanation for their previous lack of detection in the early response. Thus, the basis for the development of a heterogenous response can be observed beginning in the primary response. The mechanism of

preferential expansion of these rare clonotypes has been hypothesized to involve affinity selection following mutation to higher affinity OX-binding participants (15).

We are faced with an intriguing biological question regarding the population dynamics of Group I and Group II antibodies. It is clear that Group I antibodies dominate the primary PC-KLH response; it is also now known that Group II antibodies obtain a strong foothold and are readily observed in the memory response. The question becomes: How are these minor clonotypes expanded in a situation where the predominant antibody population constitutes >95% of the response? The initial dominance may very well result from a distinct advantage in precursor numbers in the preimmune repertoire. Alternatively, environmental antigen in the form of PC may preferentially expand Group I antibodies (T15-like) since PC-bearing pathogens in the environment are likely to be encountered frequently and may only induce the Group I specificity; this is consistent with the observation that Group I but not Group II antibodies can bind to PC on pneumococci (16). Nevertheless, antibodies not observed in the primary response represent as much as 50% of the memory anti-PC response. Returning to the 4-stage model of immune maturation discussed earlier (17) the role of affinity selection must be considered as an important control element in determining the relative expansion of antigen responsive participants. It is possible that while Group I antibodies express high affinity receptors for PC, particularly in the context of a microbial antigenic determinant, the germline encoded binding site for PC-protein may not be optimal. The presence of the phenyl moiety in PC-protein antigens may introduce new constraints on Group I-like binding sites that are distinct from those met with free PC

or PC in the context of a microbial cell wall. Hence, primary immunization with PC-KLH induces Group I antibodies and very likely stimulates Group II antibodies which may be a minor population of heterogenous composition that is undetectable during the primary response.

As the level of antigen drops following primary immunization competition for antigen may be a major element in selectively expanding high affinity clones. If Group II antibodies displayed higher affinity receptors this would be a likely period when Group II antibodies selectively increase to share an equal portion of the response with Group I antibodies. However, we have preliminary evidence that there are not significant differences in affinity between these two populations for the hapten NPPC (Dr. U. Bruderer, unpublished observations). Thus, the population shift allowing the development of Group II antibodies may not be the direct result of selective expansion during periods of limiting antigen.

An alternative explanation, however, is the following: The effect of somatic mutation has been shown to be random (18) and sequential (19,20) and the resultant amino acid changes in the combining site may enhance as well as destroy antigen binding. During the late primary response, somatic mutation begins to impose a strong effect upon the developing immune response since following nearly every cell division one of the two daughter cells is likely to have experienced a somatic mutation in one of its two expressed V region genes (17). Thus, the rate at which a clone expands depends on the affinity of that clone for antigen as well as the ability of the V region to sustain mutations (at a

rate nearly equal to the rate of cell division) without losing the capacity to bind antigen.

The intrinsic adaptability of an antibody may become a major factor in determining its survival as the response matures. Group I antibodies may express very well-fitting combining sites that are optimally designed for PC-microbe structures; combining sites that may not need further modifications. Indeed, this feature of germline encoded V gene structures that are optimally designed has been proposed to be the result of longstanding evolutionary pressure in the form of PC-containing pathogens (2,21). Furthermore, such a well-fitting combining site may be poorly designed for incurring many somatic changes, particularly in terms of recognizing different PC determinants. In some cases, Group I antibodies may be less able to accomodate changes when required to bind to PC-protein whereas binding to free PC may remain virtually unchanged. An example of such a mutant has been reported to occur in vitro (22) and may very well typify a situation that arises in vivo.

Group I anti-PC antibodies may actually reflect superior germline encoded structures against a common environmental pathogen. Such perfection may rarely be improved upon and could be prone to destruction due to somatic changes. This would predict that variants resulting in higher affinity receptors would be uncommon. It would also predict that antigen-loss mutants would occur with some frequency as the level of somatic mutation increased. The nature of the mutations found in Group I antibodies would be expected to favor silent mutations over replacement substitutions if this prediction were to hold. Group II antibodies, on the other hand, may be more able to sustain random somatic mutations without consequent loss of antigen binding. Thus as the effects of

somatic mutation become predominant, Group II antibodies exhibiting affinity for antigen equal to Group I antibodies may be allowed an opportunity to fill the void generated by the loss of those Group I antibodies that have acquired a significant diminution in affinity through a mutation.

Group II antibodies would be predicted to exhibit more mutations than Group I antibodies and the ratio of silent to expressed changes should be smaller than that of Group I antibodies. This hypothesis is testable through sequence comparison of Group I and Group II antibodies (hybridomas) obtained during early and late primary and memory responses. Group I antibodies may also be examined for loss of antigen binding during the late primary response when somatic changes may be most evident but antigen selection is not maximal. Such variants may be derived in late primary response hybridomas and detected by examining hybridomas for the expression of V_H1 mRNA in the absence of PC-protein binding. Isolation of mutants unable to bind PC-protein could be very informative regarding features necessary for antigen binding and antibody structure. While it may be the degree of adaptability of Group II antibodies which favors their expansion in the secondary response, other factors such as different intrinsic clonal expansion rates, compartmentalization of B cell subpopulations and regulatory idiotypic networks may also influence the relative contribution of the various participants.

Group II antibodies comparatively are more diverse genetically, drawing from more than 11 distinct V gene segments which may allow the overall Group II response to withstand the deleterious effects of somatic mutation more efficiently than Group I antibodies since several different clonotypes are capable of responding. The essential structures necessary

in Group II antibody binding are not yet known. There appear to be preferred V gene elements that form Group II binding sites: V_k1-3 and V_λ1 light chains and the V_HM141 heavy chain appear to play a significant role in Group II-like antigen binding. We are currently investigating the relative contribution of the sequences encoded by these genes through direct mRNA sequencing of four hybridomas known to express V_HM14 in combination with V_λ1. Comparison of these sequences may point to sequence structures necessary to Group II binding. The presence of shared mutations in these unrelated clones would argue in favor of the idea that the observed changes were meaningful and may affect selection factors such as antigen binding, V_H-V_L associations or idiotype regulation.

In addition, we have identified five hybridomas that express the V_k1-3 gene in association with either S107 or Q52 family members. Sequence analysis of these hybridomas may provide insight into the antigen binding role of this light chain which has been shown to contribute substantially to the Group II antibody response. The findings that V_k1-3 can represent as much as 80% of the Group II anti-PC serum antibody and that amino acid sequencing of Group II serum antibody yields an unambiguous V_k1-3 sequence (23) suggest an important function for this light chain in determining the character of the Group II response. Although, the finding that V_H M141 Group II can be associated with either V_k1-3 or V_λ could also be perceived as indicating that the light chains are relatively unimportant in generating the Group II phenotype.

We have found that two Group II hybridomas use the V_H1 gene ordinarily used by Group I antibodies. The V_H sequences of these proteins may prove particularly informative since these hybridomas are

unable to bind to free PC despite the use of the T15 V_H . This may be due to critical mutations in the V_H gene that leave NPPC binding unaltered but do not allow PC binding. Alternatively, such a distinct fine specificity difference may be mediated by V_k1-3 sequences. Differences in V_L usage in T15 (V_k22), M603 (V_k8) and M167 (V_k24) have been suggested to confer fine specificity differences particularly in relation to carrier determinants of the PC-immunogen (24,25). V_k1-3-associated fine specificity differences may extend these findings to include hapten-protein recognition.

Perspectives.

Due to recent advances in molecular immunology our understanding of the origins of antibody diversity is rapidly becoming complete. However, the process of diversification is known only in bare outline; the substrates have been identified but the recombinational machinery responsible for targeting the specific segments and the events that regulate their expression are not yet defined. There is accumulating evidence that this is a tightly controlled, stage and tissue-specific process (26). Analysis of different stages of B cell ontogeny has shown that the assembly and subsequent expression of V genes is non-random and appears to be position dependent (27); the mechanisms through which this may come about and its biologic function are as yet unclear but may reflect an important role in limiting the antibody repertoire early in development to insure self-tolerance in the developing immune response. Furthermore, the biased expression of certain V genes seen in very early pre-B cell stages reflects the programmed acquisition of antibody

specificities that appear at characteristic times during murine development which is replaced by an apparently random and diverse set of clonotypes in the mature animal (28). How randomization of the skewed immature population occurs is unclear but may be the biologic consequence of antigen selection or specific cell-cell interactions.

Much less is known about the molecular events that control the development of memory B cells. While specific features of the immune response can be readily ascribed to immunological memory, the identity of memory B cells remains obscure. There are no unambiguous cellular markers making the origins of these cells and their relative placement in B cell differentiation particularly difficult to assess. Since direct cellular identification is lacking, an effort has been made in our laboratory to define memory populations based on functional criteria which have resulted in the identification of distinct subpopulations that differ in their triggering requirements (29,30). An understanding of the development of these subpopulations, their relationship to one another and the functional basis and molecular consequences of their different activation requirements is essential to identifying the events that lead to differention of memory B cells.

It remains an open question whether the process of antibody diversification acts equally in all B cell populations; it has been suggested that the potential for somatic diversification is different in various B cell subpopulations (distinguishable by their activation requirements) which could guarantee the expression of unaltered germline genes in the antibody response (31). Thus, different activation requirements may reflect distinct B cell populations generated in the course of B cell maturation. Such differences could result in asymmetric

recruitment of B cell clones into the memory pool which display distinct responses to selection (such as specific activation or supression) which in turn could bias the composition and degree of heterogeneity in the memory response.

Our findings point to a very strong selection process operating during the development of immunological memory that allows new gene combinations to be recruited in the face of overwhelming clonotype dominance by another family of closely related molecules. These findings however do not allow us to say whether selection is directed by antigen or anti-idiotypic regulation, or perhaps by more complex mechanisms such as separate compartmentalization of virgin and memory lymphocytes and differential activation requirements. Nevertheless, our findings demonstrate that the memory response to PC is characterized by a shift toward new gene combinations resulting in increased binding heterogeneity which may be visualized as fulfilling a basic biological function of a mature immune response; that is, defense against a vast array of pathogens that present highly mutable targets to the developing antibody response.

REFERENCES

1. Claflin, J.L. and S. Rudikoff. 1977. Uniformity in a clonal repertoire: a case for a germline basis of antibody diversity. Cold Spring Harbor Symb. Quant. Biol. 41:725-734.

2. Crews, S., J. Griffin, H. Huang, K. Calame and L. Hood. 1981. A single V_H gene segment encodes the immune response to phosphocholine: somatic mutation is correlated with the class of the antibody. Cell 25:59-66.

3. Perlmutter, R.M. 1984. The molecular genetics of phosphocholine-binding antibodies. In The Biology of Idiotypes, Plenum Press, New York. p. 59-74.

4. Chang, S.P. and M.B. Rittenberg. 1981. Asymmetric memory to phosphorycholine in vitro. I. Asymmetric expression of clonal dominance. J. Immunol. 126:975-980.

5. Chang, S.P., M. Brown and M.B. Rittenberg. 1982. Immunologic memory to phosphorycholine. II. PC-KLH induces two antibody populations that dominate different isotypes. J. Immunol. 128:702-706.

6. Perlmutter, R.M., S.T. Crews, R. Douglas, G. Sorenson, N. Johnson, N. Nivera, P.J. Gearheart and L. Hood. 1984. The generation of diversity in phosphorycholine-binding antibodies. Adv. Immunol. 35:1.

7. Gearheart, P.J., N.D. Johnson, R. Douglas and L. Hood. 1981. IgG antibodies to phosphorycholine exhibit more diversity than their IgM counterparts. Nature 291:29-34.

8. Wicker, L.S., G. Guelde, I. Scher and J. Kenny. 1982. Antibodies from the $Lyb5^-$ B cell subset predominate in the secondary IgG response to phosphocholine. J. Immunol. 129:950-953.

9. Wicker, L.S., G. Guelde, I. Scher and J.J. Kenny. 1983. The asymmetry in idiotype-isotype expression divergence in the expressed repertoires of $Lyb5^+$ and $Lyb5^-$ B cells. J. Immunol. 131:2468-2476.

10. Wysocki, L., T. Manser and M.L. Gefter. 1986. Somatic evolution of variable region structures during an immune response. Proc. Natl. Acad. Sci. 83:1847-1851.

11. Manser, T., S.-H. Huang and M.L. Gefter. 1984. Influence of clonal selection on the expression of immunoglobulin variable region genes. Science 226:1283-1288.

12. Kaartinen, M., G.M. Griffiths, A.F. Markham and C. Milstein. 1983. mRNA sequences define an unusually restricted IgG response to 2-phenyloxazolone and its early diversification. Nature 304:320-324.

13. Berek, C., G.M. Griffiths and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. Nature 316:412-418.

14. Kaartinen, M., J. Pelkonen and O. Makela. 1986. Several V genes participate in the early phenyloxazolone response in various combinations. Eur. J. Immunol. 16:98-105.

15. Milstein, C., J. Even and C. Berek. 1986. Molecular events during the onset and maturation of the antibody response. Biochem. Soc. Symp. 51:173-182.

16. Chang, S.P., R.M. Perlmutter, M. Brown, C.H. Heusser, L. Hood and M.B. Rittenberg. 1984. Immunologic memory to phosphocholine. IV.

Hybridomas representative of Group I (T15-like) and Group II (non-T15-like) antibodies utilize distinct V_H genes. J. Immunol. 132:1550-1555.

17. Manser, T., L.J. Wysocki, T. Gridley, R.I. Near and M.L. Gefter. 1985. The molecular evolution of the immune response. Immunol. Today 6:95-101.

18. Clarke, S.H., K. Huppi, D. Ruezinsky, L. Staudt, W. Gerhard and M. Weigert. 1985. Inter- and Intraclonal diversity in the antibody response to influenza hemagglutinin. J. Exp. Med. 161:687-704.

19. McKean, D., K. Huppi, M. Bell, L. Staudt, W. Gerhard and M. Weigert. 1984. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. Proc. Natl. Acad. Sci. USA 81:3180-3184.

20. Sablitzky, F., G. Wildner and K. Rajewsky. 1985. Somatic mutation and clonal expansion of B cells in an antigen-driven response. Embo. J. 4:345-350.

21. Perlmutter, R.M., B. Berson, J.R. Griffin and L. Hood. 1985. Diversity in the germline antibody repertoire. Molecular evolution of the T15 V_H gene family. J. Exp. Med. 162:1998-2016.

22. Cook, W.D., S. Rudikoff, A.M. Giusti and M.D. Scharff. 1982. Somatic mutation in a cultured mouse myeloma cell affects antigen binding. Proc. Natl. Acad. Sci. USA 79:1240-1244.

23. Bruderer, U. 1985. Regulation of antibody isotype expression in mice. Inauguraldissertation philosophisch-Naturwissenschal-ftlichen Facultat. der Univesitat Basel.

24. Leon, M.A., N.M. Young. 1971. Specificity for phosphorycholine of six murine myeloma proteins reactive with Pneumococcus C polysaccharide and βlipoprotein. Biochemistry 10:1424-1429.

25. Andres, C.M., A. Maddalena, S. Hudak, N.M. Young and J.L. Claflin. 1981. Anti-phosphocholine hybridoma antibodies. II. Functional analysis of binding sites within three antibody families. J. Exp. Med. 154:1584-1598.

26. Yancopoulos, G.D. and F.W. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. Cell 40:271-281.

27. Yancopoulos, G.D., S.V. Desiderio, M. Paskind, J.F. Kearney, D. Baltimore and F.W. Alt. 1984. Preferential utilization of the most J_H-proximal V_H gene segments in pre-B-cell lines. Nature 311:727-733.

28. Perlmutter, R.M., J.F. Kearney, S.P. Chang and L.E. Hood. 1985. Developmentally controlled expression of immunoglobulin V_H genes. Science 227:1597-1600.

29. Tittle, T.V. and M.B. Rittenberg. 1980. IgG B memory cell subpopulations: Differences in susceptability to stimulation. J. Immunol. 124:202.

30. Tittle, T.V. and M.B. Rittenberg. 1978. Distinct subpopulations of IgG memory B cells respond to different molecular forms of the same hapten. J. Immunol. 121:936.

31. Woodland, R.T. and B. Huber. 1984. Selective activation by thymus independent antigens of distinct B cell subpopulations expressing a major cross-reactive idiotype. J. Immunol. 133:1801-1809.

Figure 1. Extensive antibody diversity is displayed in the anti-PC memory response. The V_H (bottom) and V_L (left) gene combinations that generate Group I (●) or Group II (■) antibodies are plotted. Anti-PC antibodies sharing V gene elements with primary antibodies (Group I) in association with new V genes observed in the memory response are indicated by the hatched symbols (◍, Group I; ◪, Group II). M141, M141* and M141** are related but distinct members of the Q52 V_H gene family. V12 is a member of the J558 V_H family; V11 and V1 are members of the S107 V_H family. The V_L genes listed all derive from separate families.

ANTIBODY DIVERSITY IN THE ANTI-PC MEMORY RESPONSE