IMMUNOLOGIC MEMORY TO PHOSPHORYLCHOLINE

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

STATEMENT OF PROBLEM

The antibody response to simple, chemically-defined haptens has served as an extremely useful model in studying the immune system. Such models have allowed us to begin to understand the nature of the antigen-combining site of antibody molecules, to define the specificity and heterogeneity of antibody populations, and to follow the shifts in antibody populations expressed in the course of an antibody response. A hapten which has been particularly useful is phosphorylcholine (phosphocholine, PC), an antigenic determinant which is widespread in nature (1). PC was first recognized as an immunological tool when it was noted that several spontaneous BALB/c myelomas specifically bound this haptenic determinant as part of the C polysaccharide of Streptococcus pneumoniae (2-5). It was subsequently found that BALB/c mice immunized with PC-containing antigens produced antibodies with combining sites serologically related to several of the spontaneous PC-binding myelomas (4,6,7). These discoveries were soon followed by extensive studies on the PC model system dealing with such diverse topics as the genetic control of variable region expression, cellular interactions influencing variable region expression, and the molecular basis of antibody diversity.

In our laboratory, the PC system offers a model to study variable region expression of antibodies involved in the memory response. Previous studies had concentrated on the in vitro antibody response to trinitrophenyl (TNP) antigens which elicit potent IgG memory in mice (8). Using this model, memory B cell subpopulations responsive to

T cell dependent and T cell independent forms of TNP were defined (9), analogous to previously defined primary B cell subsets (10). While memory B cell subsets could be clearly demonstrated by functional criteria, it was important to determine variable region expression of functionally separate subsets. The extensive variable region heterogeneity of the anti-TNP response made it extremely difficult to approach such a question with this system, and the memory PC system was developed to deal with this question since the anti-PC antibody response appeared to be less complex than most other anti-hapten responses.

The objectives of this thesis project were:

(1) To determine the variable region heterogeneity of the memory anti-PC response induced by immunization with PC-KLH as measured by

(a) fine specificity for PC analogs, and

(b) expression of the T15-idiotype

and to compare the heterogeneity of the memory antibody pool to that of the primary response.

(2) To determine whether differences in variable region expression exist among the various immunoglobulin classes expressed in the memory response (IgM, IgA, IgG1, IgG2a, IgG2b and IgG3).

(3) To determine the origin of novel variable regions expressed in the memory response but undetectable during the primary response.

INTRODUCTION AND LITERATURE REVIEW

General properties of the anti-phosphorylcholine response.

The anti-PC antibody response is restricted in heterogeneity in several mammalian species (11). The most extensively studied model for this antibody response has been the BALB/c mouse because its antibody response to PC is dominated by a single idiotype defined by the PC-binding myeloma protein TEPC 15 (7,12,13). Since all individuals of this and several other mouse strains express the T15 idiotype, it was proposed that this variable region is encoded in the germ line (6). This has recently been confirmed by analysis of homologous heavy and light chain variable region sequences in the mouse genome (14). Genes encoding this variable region appear to be highly conserved in evolution since a human Waldenströms macroglobulin was isolated which appears very similar in amino acid sequence (15).

Structure of anti-PC antibodies.

A large body of information is available on the structure of anti-PC antibodies because of the availability of a collection of PC-binding BALB/c myelomas closely related to natural anti-PC antibodies of BALB/c. These IgA(k) myelomas have been independently isolated in laboratories at the Salk Institute and the National Institutes of Health since 1967 (2-4) and specifically bind the C-carbohydrate of pneumococci (3-5). C-carbohydrate binding was inhibited by free phosphorylcholine, establishing that PC is the relevant antigenic determinant of the carbohydrate (16). Studies using myeloma-specific (anti-idiotype) antisera (4,5) and N-terminal amino acid

sequencing of heavy and light chains of these myeloma proteins (17,18) identified a set of closely-related, independently-derived PC-binding myelomas referred to as the T15-group (S63, T15, H8, S107 and M299) along with another set of idiotypically distinct PC-binding myeloma proteins (M511, M603, and M167). Myeloma proteins expressing the same idiotype and N-terminal heavy and light chain sequences shared fine specificity patterns for hapten analogs of phosphorylcholine, while idiotypically distinct PC-binding myeloma proteins also differed in fine specificity (19). However, all of the PC-binding myeloma proteins possessed closely related V_H sequences and were categorized into the V_H-4 subgroup, and were associated with three distinct V_K sequences: V_K-22 (T15 group), V_K-24 (MOPC 511, MOPC 167) and V_K-8 (McPC 603) (18,19).

The three-dimensional structure of the PC antigen binding site was analyzed by X-ray crystallography (20). The binding cavity is formed by five of the hypervariable regions: L1, L3, H1, H2 and H3. Hapten contacting residues were identified on both V_H and V_L, and V_H:V_L interactions maintain the structure of the binding site (21). Phosphorylcholine occupies a small part of the 12Å X 15Å X 20Å binding cavity and is bound asymmetrically, being closer to the H chain than the L chain. The positively charged choline group interacts with glu-35 (H chain), glu-59 (H chain) and leu-96 (L chain) while the negatively charged phosphate group interacts with arg 52 (H chain), lys 54 (H chain) and tyr 33 (H chain). These contact residues are conserved in all of the PC-binding myeloma proteins (21).

Recent analyses of antibody gene structure have resulted in the division of the heavy and light chain variable regions into separate genes since they exist as discontinuous segments in the DNA of undifferentiated cells: V_H-D_H-J_H and V_L-J_L for heavy and light chains, respectively (22-24). Studies on light chain sequences reveal a uniform expression among all PC-binding myeloma proteins of J_K-1, which encodes the V_L contact residue leu-96 (25). Expression of J_H was also conserved, with all PC-binding myeloma proteins utilizing J_H-1, while D_H segment sequences differed (26).

TEPC 15-related antibodies have been detected in all normal mouse strains examined although in some strains this idiotype was not dominant (6,7,13). Allelic forms of TEPC 15-like anti-PC antibodies were demonstrated by a non-binding site anti-idiotype (27) and by V_H sequence analysis (28,29). The prototype allele of TEPC 15 was the PC-binding myeloma protein C3 induced in congenic CBB-22 mice which have a C57BL Igh allotype locus (Igh^b) on a BALB/c background (29). The BALB/c-T15 allele differs from the C57BL/6-C3 allele at four positions in the V_H framework (amino acids 14, 16, 40 and 44) and one position in the J_H segment (amino acid 109). This allelism may have developed before the evolution of C_H alleles or independently of the C_H alleles since expression of T15 or C3 alleles does not correlate with Igh haplotype (29).

Isoelectric focusing of PC-immunoabsorbent purified antibodies of BALB/c mice immunized with PC-KLH produced T15 idiotype positive patterns of limited heterogeneity for all IgG subclasses (30). These results were interpreted as supporting a highly conserved BALB/c

anti-PC response restricted to a very few or possibly a single germline V_H gene in combination with the different IgG C_H genes. Other inbred mice appeared to express strain-characteristic isoelectric focusing banding patterns, with T15 idiotype positive antibodies being a major species in most strains (31). However, occasional bands which were T15 idiotype positive but atypical in pI were detected at low frequency (∿10%) in BALB/c. These T15-related but non-identical antibodies were detected especially in neonatally T15-idiotype suppressed BALB/c mice and were occasionally IgG3 but usually IgG1. An increased frequency of these antibodies were noted in certain other inbred strains, with some mice of the Igh^j haplotype having the highest frequency, and also in wild Mus musculus (32,33). Two strains congenic to BALB/c also expressed high frequencies of these bands, suggesting that the capacity for a heterogeneous T15-positive response may be affected by the H-2 (or a closely-linked) locus (33). Therefore, the potential to generate diverse T15 idiotype positive antibodies exists but has not been readily detected in BALB/c and many other commonly used strains. The basis for this diversity has not been studied, but it is likely that different V_H-D_H-J_H or V_L-J_L combinations and/or somatic mutation of V genes are involved.

In addition to T15 idiotype positive antibodies, the murine PC-specific antibody response has also been shown to include antibodies bearing M511- and M603-defined idiotypes. Although rare in BALB/c, these idiotypes can be elicited as major species in other strains depending somewhat on the choice of immunogen. For example, C57BL/6J mice produced approximately equal amounts of antibodies cross reactive

with T15, M511 and M603 anti-idiotypes upon immunization with PC-KLH (34). In contrast, immunization with the Streptococcus pneumoniae strain R36A vaccine (R36A) elicited a smaller M511 idiotype positive response in this strain (34). The structural heterogeneity of M511 idiotype positive antibodies in various strains has been analysed by isoelectric focusing (35). M511 idiotype positive antibodies can be detected in both IgG1 and IgG3 subclasses and the isoelectric focusing patterns of both subclasses is more complex than T15 idiotype positive patterns. Multiple band subsets representing at least four groups in the IgG1 subclass and five to six in the IgG3 subclass are observed regularly for SEC and C57L mouse sera. These results suggest a greater heterogeneity among M511 idiotype positive antibodies which appear to express a common VK-24 L chain but may differ in V_H-D_H-J_H gene segments. A somatic mutation basis for this heterogeneity is considered unlikely since complex isoelectric focusing patterns overlap among individuals of the same strain, but this mechanism has not been formally excluded.

Anti-PC antibodies cross reactive with the McPC 603 idiotype can be detected in some strains after immunization with PC-KLH or R36A, however, immunization with Proteus morganii (Potter), a strain Potter isolated from the mouse intestine, elicits primarily M603 idiotype positive antibodies even in BALB/c (36). T15 idiotype positive antibodies are usually detected early in the primary response to P. morganii (Potter) but are succeeded and replaced by T15-idiotype negative antibodies later in the primary and throughout the secondary response. Of all the PC-binding myeloma proteins only McPC 603 binds

P. morganii (Potter). While these antibodies contain light chains which cofocus with the McPC 603 (VK-8) light chain in pH gradients, anti-idiotypic antibodies raised against purified McPC 603 protein do not cross-react with these antibodies. On the other hand, a heterologous binding-site specific anti-idiotype raised against a pool of anti-PC antibodies from mice immunized with P. morganii (Potter) cross-reacts with McPC 603. It was considered unlikely that this anti-idiotype was V_L-specific since it did not bind W3207, another PC-binding myeloma protein which contains the V_K-8 light chain. W3207 differs from McPC 603 at the heavy chain variable region D_H segment, sharing most of the V_H-4 sequence (with only three amino acid interchanges between the two proteins) and all of the J_H sequence, and thus it is possible that the McPC 603-cross-reactive idiotypic determinant is determined by the D_H sequence. It should also be noted that unlike McPC 603, W3207 does not bind P. morganii (Potter), suggesting an important role of the D_H segment in conferring its unique specificity to McPC 603. However, only when the entire V_L sequences are determined for both proteins will it be possible to confirm the major role of D_H in the specificity and idiotype of McPC 603 and of antibodies induced by P. morganii (Potter).

Analysis of isoelectric focusing patterns of McPC 603 cross-reactive antibodies indicated that these anti-PC antibodies were also considerably more heterogeneous than $T15^+$ antibodies (37). The major isotype elicited by P. morganii (Potter) was IgG3, although IgG1 was also a significant component of the response. IgM antibodies were only minimally detected and IgG2 was undetectable. Isoelectric

heterogeneity was observed for both BALB/c (38 distinct banding patterns) and A/HeJ (19 distinct banding patterns) antibodies. Most of these bands were positive for the McPC 603 cross-reactive idiotype; however, since this anti-idiotype was raised against a pool of anti-PC antibodies rather than a single myeloma protein it is possible that it is specific for more than one V_H-V_L pair of anti-PC antibodies. Since some bands of different individuals appear to cofocus it was proposed that McPC 603 cross-reactive antibodies represent a set of closely related proteins whose diversity is encoded in the germline and may be determined by different V_H-D_H-J_H combinations. The anti-Proteus morganii (Potter) response is therefore one situation where greater heterogeneity in V region expression occurs in the BALB/c anti-PC response. As we shall see in the PC-KLH memory response, however, such heterogeneity can be obtained with antigens which characteristically elicit T15 idiotype-dominated primary responses.

Research aimed at characterizing the diversity of antigen-specific antibody responses has been revolutionized within the last five years by the hybridoma technology developed by Kohler and Milstein (38). This approach has been used in combination with recombinant DNA techniques to define the genes encoding the variable regions of anti-PC antibodies, and to assess the relative contributions of multiple germline genes, somatic mutation, somatic recombination and combinational joining to the overall diversity of an antibody response.

Anti-PC hybridomas have been generated using spleen cells of mice immunized with the pneumococcal vaccine R36A (39,40) or PC-KLH (26,40). The overall results of these hybridoma analyses indicate that a major

part of the response to PC is comprised of antibodies related to the three groups of PC-binding myeloma proteins: TEPC 15 (VH-4, VK-22), MOPC 511 (VH-4, VK-22), and McPC 603 (VH-4, VK-8); however, a minority of hybrids expressing different V_H and/or V_L regions were detected in mice suppressed for the T15-idiotype (39). Also even among those hybridoma antibodies utilizing the major V_H and V_L groups there is considerable variation in amino acid sequence (26). Our own work will demonstrate that different V regions can constitute a major proportion of the memory antibody response of normal BALB/c mice immunized with PC-KLH.

Hybridomas derived from R36A hyperimmunized BALB/c were predominantly of the IgM isotype, although a few IgG hybridomas were reported (31). The high incidence of IgM anti-PC hybridomas is often disproportional to the level of IgM relative to other immunoglobin isotypes in serum. This phenomenon is not understood, but may be due to an increased fusing efficiency of IgM-producing B cells. Most of these anti-PC hybridomas were related to TEPC 15 in idiotype and N-terminal sequences of V_H and V_L. Two BALB/c IgM hybridomas obtained by R36A immunization expressed the cross-reactive idiotype of McPC 603 and their light chains cofocused with that of McPC 603 (40). Fusion of T15-idiotype suppressed BALB/c spleen cells from mice immunized with R36A resulted in the production of hybridomas that either were cross-reactive (but non-identical) for the T15 idiotype or completely non-cross-reactive for the T15 idiotype (39). N-terminal amino acid sequences of these T15-cross-reactive hybridomas indicated the expression of V_H-4 in combination with V_L sequences homologous (but not

necessarily identical) to V_K-22, V_K-8 and V_K-24 of the prototype myeloma proteins (41). Hybridomas non-cross-reactive for the T15 idiotype expressed a V_K-24-like sequence in combination with a V_H sequence clearly distinct from V_H-4 (homology to V_H-4 of first 37 amino acids was 46%) (41,42). This sequence was designated V_H-12 and appears to differ from all V_H groups defined previously, although it is 76% homologous to V_H-7 (α(1→3)dextran) binding antibodies MOPC 104E and J558) (42). The detection of these hybridomas suggests that V regions distinct from T15 exist, some of which may express V_H sequences encoded by gene segments other than V_H-4. It is possible that the TEPC-15 distinct V regions we observe in the memory response to PC-KLH are related to such novel V_H sequences.

Hybridomas generated with spleen cells of PC-KLH immunized BALB/c mice express high levels of T15-idiotype negative antibodies associated with V_K-24 and V_K-8 L chains along with T15-idiotype positive, V_K-22 antibodies even in the absence of idiotype suppression (26,40). IgG hybridomas are more frequent with PC-protein immunization, consisting primarily of IgG1 and IgG3. One set of hybridomas elicited by PC-KLH exclusively expressed the V_H-4 sequence, although one IgG1 sequence did differ at four framework and two HV-1 positions within the first 36 amino acids, theoretically making it eligible for designation as a distinct V_H group (26). Complete amino acid sequencing of the V_H region was conducted for five IgM hybridomas and five IgG hybridomas (26). Among the IgM hybridomas positions 1-95, corresponding to the V_H gene segment, were identical to TEPC 15 (V_H-4) as were positions 110-125, spanning the J_H segment (J_H-1), while positions 96-109

encompassing D_H and its junctions with V_H and J_H were variable in 2/5 cases. In contrast, multiple amino acid interchanges were commonly detected among IgG1 and IgG3 hybridomas, although these still were highly homologous to V_H-4. The J_H segment of all IgG hybridomas was identical to J_H-1, and invariant, while the D_H segment and its junctions were variable. Amino acid interchanges relative to T15 have also been reported for several PC-binding IgA myeloma proteins, primarily localized to V_H and D_H (43-45). These observations were the basis for the theory that somatic mutation may be coupled to the class switch event/mechanism (to be discussed later).

Fusions were also performed with spleen cells of other mouse strains immunized with PC-KLH, including those which do not display T15 idiotype dominance (C57L) and those which express IEF heterogeneity of their T15 idiotype positive antibodies (CBA, BALB.G, PL) (46). All strains generated examples of hybridomas expressing idiotypes defined by T15, M511 and M603 proteins. However, when expression of the T15 idiotype was compared for hybridomas of various mouse strains it was apparent that idiotype expression was extremely variable, and in many cases the hybridomas appeared cross-reactive rather than identical to T15. In agreement with the normal presence of T15, M511 and M603 idiotype positive antibodies in their immune serum, CBA hybridomas were found which were either T15 idiotype-positive, M511 idiotype-positive, or M603 idiotype-positive, and C57L hybridomas positive for the M511 idiotype or M603 idiotype were obtained. The light chains of these hybridomas often cofocused in isoelectric focusing with the three major

V_K groups, however, there were several exceptions which may represent distinct light chains (40,46).

The binding specificity of PC-KLH and R36A hybridomas was examined by two approaches which appear to define distinct subsites within the binding cavity of anti-PC antibodies: (1) measurement of fine specificity of the hapten-binding site using a variety of PC hapten analogs, and (2) measurement of the binding of various PC-antigens possessing different linkage groups and carrier determinants presumably involving a separate subsite within the binding cavity (46). Hybridomas expressing the T15 idiotype were very similar to each other in specificity for the various PC analogs, displaying highest avidity for phosphorylcholine and 3-(p-azophenyl-phosphorylcholine)-N-acetyl-YGG Boc hydrazide (PC-Y). However, carrier specificity differed among T15 idiotype positive hybridomas; while all bound a PC-protein conjugate equally well, binding of S. pneumoniae and P. morganii (Potter) was variable. The detection of P. morganii (Potter) binding among T15 idiotype positive hybridomas was unexpected since TEPC 15 does not bind this bacterium. It is possible that the variable region segment responsible for P. morganii (Potter) binding does not overlap with the site recognized by the anti-T15 idiotype. These hybridomas express the VK-22 light chain and not the VK-8 light chain associated with McPC 603, consistent with the hypothesis that P. morganii (Potter) binding may not be determined by V_L but rather by D_H as discussed earlier. It has been noted that D_H is highly variable among T15-idiotype positive antibodies, and a portion of these antibodies may utilize the D_H required for P. morganii (Potter) binding.

M603 cross-reactive idiotype positive hybridomas differed in hapten specificity from T15 idiotype positive hybridomas (46). Most M603 cross-reactive idiotype positive hybridomas displayed highest avidities for PC, PC-Y and 3-carboxypropyl TMA (3COOH). Several but not all of these hybridoma antibodies bind P. morganii (Potter). One exceptional hybridoma of this group had a very low avidity for PC and was significantly inhibited only by PC-Y; this hybridoma possesses a light chain distinct from that of McPC 603 and does not bind P. morganii (Potter). This hybridoma may be related to the group II antibodies defined in our own studies that have a much higher avidity for p-nitrophenyl phosphorylcholine than for phosphorylcholine.

In contrast to the sharing of specificities within a group of T15 idiotype and M603 idiotype hybridomas, M511 idiotype hybridomas characteristically displayed unique fine specificity patterns when tested with the various hapten analogs. Antigen binding in this group was greatest for PC-protein conjugates. Binding of S. pneumoniae was usually low despite the fact that some of these hybridomas originated from R36A-immunized mice. Negligible binding of P. morganii was obtained. One of these hybridomas uses a V_H sequence related to two V_H gene segments distinct from that used by TEPC 15. This V_H sequence may be the product of gene conversion since it appears to utilize gene segments encoded by three members of the T15 V_H gene family (47); this hybridoma also expresses a different J_H segment (J_H-3) in combination with a V_K-24 L chain (46,47). Thus, analyses of both serum antibodies and hybridoma antibodies indicate that the diversity among M511

idiotype-positive antibodies is great and amino acid sequencing of these hybridoma proteins should reveal the basis of this diversity.

A major portion of the antibody response to PC appears to be encoded by a single germline V_H gene, called V1, whose protein product would be identical in amino acid sequence to TEPC 15 and highly homologous to all antibodies belonging to the V_H-4 group (48). The likelihood that V_H-4 proteins were encoded by a single V_H germline gene was based on nucleotide sequencing analyses of cloned DNA fragments selected from a mouse germline (sperm) library by their ability to bind to a cloned S107 (T15-identical) V_H probe (48). The S107 probe identified four major hybridizing segments of germline DNA corresponding to four distinct genes sharing 86-96% nucleotide sequence homology: V1, V3, V11 and V13. These genes were defined as the T15 gene family. Four additional clones less homologous to the S107 V_H probe ($\sim$75% homology) were detected and designated as non-members of the T15 family (48). A comparison of nineteen anti-PC V_H region amino acid sequences (myeloma and hybridoma) to the sequences encoded by V1, V3, V11 and V13 indicated that each of these expressed V_H regions, including nine regions which were variant in sequence, was most homologous to V1. Nucleotide sequences indicated that at least expressed genes of three myelomas and an hybridoma (M603, T15, M167, and HPCM2) also shared 5' flanking sequence with V1. Although V11 and V13 are highly homologous to V1, it is possible that these proteins encode non-PC binding antibodies although an anti-PC hybridoma has recently been reported which may be the product of gene conversion of three genes within the T15 V_H gene family (47). The V11 sequence is used by one known myeloma tumor

M47A; however, the specificity of this myeloma is unknown (47). The V3 sequence appears to be a pseudogene, a common occurrence among multigene families, by virtue of 3 mutations preventing its expression: (1) an in-frame stop codon, (2) a four base insertion which brings a termination codon into the translational reading frame, and (3) modified (and presumably non-functional) V_H-D_H joining recognition elements (49).

As already mentioned, in the amino acid sequence study of 19 V_H regions of anti-PC myelomas and hybridomas 9 variant sequences were obtained (26). Each variant sequence was observed only once and analysis of substitutions at 24 positions resulted in only one substitution explainable by gene conversion or somatic recombination within the T15 V_H gene family (26). A more likely explanation for sequence variation was a somatic mutation mechanism which appeared to be correlated with the class switch from production of IgM to IgG or IgA antibodies. This mechanism appears to act in a <u>trans</u> manner since it can affect both V_H and V_K sequences; such a mechanism presumably would involve diffusible enzymes induced or activated (derepressed) by the class switch event. The patterns of somatic variation have been intensively studied for two myelomas, M167 and M603. Somatic variation in these V_H regions is quite extensive, ranging from 1.4% for M603 to 3.8% for M167, and includes both coding regions and adjacent flanking regions. Nucleotide sequence variation appears to be sharply localized in and around the V_H gene, decreasing significantly at 2.3kb downstream from the rearranged V_H region and non-existent at 5kb on either side of the V_H gene as well as in the co-expressed C_H gene. Many

silent as well as replacement substitutions occur in variant V_H gene coding regions, and small deletions and insertions occur as well as substitutions. The selection and expansion of these variant B cells has been postulated to result from antigen selection or selection (positive or negative) by the idiotype-anti-idiotype regulatory network. It has been reported that some IgG variants display a higher affinity for PC-protein conjugates than TEPC 15 (50). With respect to the idiotype network theory, idiotype-specific T cells appear to recognize both V1-identical and variant anti-PC antibodies (51). Although the association of the somatic mutation mechanism with the class switch is controversial due to an apparently latent heterogeneity of IgM antibodies in the anti-PC response (41,52), it is becoming clear that somatic mutation is an important means of generating diversity in the antibody response.

In summary, the structure of anti-PC antibodies has been studied using a variety of experimental approaches. These studies have shown that while a major portion of the anti-PC response involves the heavy and light chain immunoglobulin regions defined by the TEPC 15 PC-binding myeloma protein, the antibody response to this antigenic determinant is significantly heterogeneous. The most subtle variants are antibodies which differ at point mutations from TEPC 15 but still derive from the same set of germline V genes (26). At the next level of variation are those antibodies which still utilize the characteristic V_H-4 sequence encoded by the V1 gene, but in combination with different V_K sequences, i.e. V_K-24 (MOPC 511) or V_K-8 (McPC 603) (17,19,26,35,36,40). Additional complexity is introduced with

antibodies which utilize the heavy chain V-1 gene combined with different D_H and, possibly, J_H genes (36); in parallel there may be different combinations of V_K-J_K genes. The most extreme variants, however, are antibodies which utilize V_H genes distinct from the V1 gene (41,42,47). These genes may be derived from other members of the T15 V_H gene family, and as in the example presented earlier may result from gene conversion within a family (47); alternatively these genes may come from unrelated V_H germline genes (41,42).

Variant antibodies are prominent in mouse strains other than BALB/c (32,33,35,37), but are also produced by BALB/c depending on the immunogen (36) and on whether the expression of the T15 idiotype has been suppressed (39). Moreover, as we will show in our own studies, such variant antibodies constitute a major portion of the memory response to PC antigen and are asymmetrically distributed among the various immunoglobulin classes.

Ontogeny of the anti-PC response.

An ordered sequence of development of antigen-specific B cells has been observed during neonatal development in the mouse which appears to be genetically programmed (53). While splenic B cells specific for dinitrophenyl (DNP) are present at high frequency at birth, splenic B cells responsive to PC are not detected until day 6-7 after birth (54). Provision of adult accessory cells and carrier-primed T helper cells does not accelerate the appearance of an anti-PC-protein response, confirming that it is the B cell which is unresponsive in neonatal mice. This late appearance of PC-specific B cells is influenced by the form of the antigen, however, since this observation is made only

if the immunogen is PC-protein or R36A (54,55). Immunization with PC-lipopolysaccharide (PC-LPS) elicits a significant PC-specific response as early as 1 day after birth (55). Although initial studies seemed to rule out the early sequestration of PC-protein responsive B cells in fetal liver or neonatal bone marrow (54), recent studies on 1 day old liver indicate that PC-protein responsive B cells are lodged at this site at this stage of development and only later migrate to the spleen (55). Sublethal irradiation experiments indicated that the neonatal liver contains immature PC-committed progenitor cells absent in the adult lymphoid system (56). These progenitors are relatively radioresistant and differentiate into PC-responsive mature B cells faster than uncommitted stem cells. The T15 idiotype can be detected among the earliest appearing B cells responding to PC-LPS, however, this idiotype does not achieve dominance over other PC-specific clones until 6-10 days after birth (55).

The presence of environmental PC antigens during early B cell development exerts long-term effects on the quality of the anti-PC response in adult mice (57). In a series of experiments where BALB/c mice were maintained germfree for 7-12 months before colonization with selected commensal bacteria and subsequent transfer to a conventional environment, a depressed level of T15 idiotype expression was observed in spleen, mesenteric lymph nodes and Peyer's patches even at 8 months after colonization or conventionalization. Prevention of exposure to normal environmental antigens permanently altered idiotype expression in these mice, suggesting that antigen selection of neonatal B cells irreversibly affects idiotype expression even in animals which appear

to be genetically predisposed to express a particular idiotype in a conventional environment.

The mechanism(s) of clonal dominance.

The antibody response of BALB/c mice to phosphorylcholine is dominated by the T15 idiotype despite the fact that BALB/c mice have the potential to generate a more complex response to PC (58). In addition to making up the majority of serum antibody and PFC responses (6,7,12), the T15 clone has also been shown to predominate at the B cell precursor level (58,59), and T15 dominance is seen when the antibody response in specifically induced by antigen (6,7,12) or by direct trigerring of B cells by T helper cells in the absence of antigen (60). Consequently, a central question raised by these observations has been what is the mechanism by which a single clone of B cells comes to dominate the antibody response to this antigen?

Several laboratories have noted that events during the neonatal period appear to be critical for the establishment of T15 dominance (54,61). Kaplan et al. have postulated that the selection of the dominant clones occurs during this period and is dependent on interactions of the T15 idiotype positive B cell progenitor with the neonatal environment (61). The B cell progenitor is a relatively immature cell type which undergoes rapid renewal in the neonate, appears to be less sensitive to irradiation (which may reflect its ability to repair radiation damage), and is progressively lost with age (55,62). A crucial role of the neonatal environment is supported by the establishment of dominance when neonatal liver cells were adoptively transferred

to lethally irradiated neonatally suppressed hosts but not to normal adult hosts (61).

The emergence of T15 dominance during ontogeny of the anti-PC response can be followed when appropriate immunogens are utilized and when particular lymphoid organs are examined (55). Neonatal animals immunized with PC-LPS generate anti-PC splenic PFC by day 1; however, their PFC are not dominated by T15 until day 10-12. While the earliest PC-KLH splenic PFC responses are already T15 dominant, this response also goes through a non-dominant phase early in development (55). Idiotype analysis of 1 day old neonatal liver PC-KLH responsive precursors reveals that these precursors are not T15 dominant although as early as on day 6 T15 idiotype positive precursors dominate in the spleen. Thus, specific antigen-sensitive precursors mature before the onset of T15 dominance and the timing of these events may differ for B cell subpopulations responding to different forms of the PC antigen. This early phase in clonal development is also characterized by other unique properties which may be of relevance to the establishment of clonal dominance. The T15 idiotype positive response is extremely sensitive to anti-idiotype suppression during the neonatal period; a single injection of anti-T15 antibodies at birth produces complete and long-term suppression of T15 antibodies while a similar treatment of adult mice has only transient effects on T15 expression (63). Low levels of autologous neonatal anti-idiotype can be detected in the serum of neonatal BALB/c mice on days 9-11 after birth (64). This transient production of anti-idiotype may implicate a role for an idiotype network in affecting clonal dominance during this period.

In addition to properties of the immune system which appear to play a role in establishing T15 dominance, there is evidence that interactions of environmental antigens with the neonatal immune system may also be of importance. Studies on the anti-PC response of germ-free BALB/c mice selectively colonized with specific commensals indicate that neonatal exposure to PC-containing environmental antigens which do not stimulate the T15 clonotype result in an anti-PC response which is not dominated by the T15 idiotype and which persists even after these mice were conventionalized as adults (65).

In addition to regulatory mechanisms being responsible for clonal dominance, it has been suggested that antibody avidity may also determine whether a clone is dominantly expressed. Etlinger et al. have found that T15 idiotype positive PFC are of higher avidity for the PC hapten than T15 idiotype negative PFC in BALB/c mice (66). In contrast, in mouse strains where T15 idiotype positive and negative clones are equally expressed, such as C57BL/6 mice, they find that the avidities of the two clonal populations are similar. Other investigators have examined the avidity of T15 idiotype positive and negative PFCs and have failed to note any difference (67), however, these latter studies were performed on mice where responses were not T15 idiotype dominant and thus would not be expected to display the avidity differences noted for BALB/c. These investigators propose that in BALB/c mice the higher binding constant of T15 idiotype positive PC-specific receptors and the presence of PC as a component of organisms in the normal flora in combination influence the frequencies of T15 idiotype positive cells.

Components of a potential regulatory idiotype network have been identified in the PC system. As already mentioned, 3 day old BALB/c mice transiently possess low levels of autogeneous anti-idiotype specific for T15 (64), and it has been suggested that these anti-idiotype antibodies may be required for expansion of this clone. These anti-idiotypic antibodies are produced during in vitro culture of neonatal B cells and are of the IgM class, supporting the interpretation that they are actively synthesized by neonatal cells rather than being of maternal origin. Hyperimmunization of BALB/c mice with PC antigens also results in the production of auto-anti-idiotype (68). Exposure of T15-dominant spleen cells to anti-idiotype in vitro results in the induction of T15 idiotype positive anti-PC antibodies (69). This mode of activation requires helper T cells recognizing the Fc moiety of the anti-idiotype, and even greater number of T15 idiotype positive PFC are obtained when antigen (R36A) is added.

T suppressor cells expressing the T15 idiotype were induced by neonatal injection of BALB/c with anti-T15 idiotype or the C polysaccharide of the R36a pneumococcus (69). Similar suppressor cells could also be stimulated in vitro with adult cells by exposure to both R36A antigen and anti-idiotype (70) or R36A alone (71). These suppressor cells were believed to act on PC-specific B cells directly, although the PC antigen may serve as an intermediary in the interaction of these two surface T15 idiotype positive cell types. Adult BALB/c mice treated with anti-T15 idiotype also generate T suppressor cells which suppress the activity of T helper cells involved in a thymus-dependent response to PC-protein antigens (72). The specificity of suppression

in this system was carrier-related, requiring challenge with the same carrier, but not carrier specific, since it acted only on a PC-carrier response and not on a TNP-carrier response. Cloned T15-positive T suppressor hybridomas have been generated by priming BALB/c mice with PC-mycobacterium which secrete PC-specific suppressor factor acting upon both IgG and IgE andi-PC responses (73).

Idiotype-specific amplifier or helper T cells were found to be necessary for a cyclical idiotypic and anti-idiotypic response generated in BALB/c mice immunized with R36A (74-76). Asynchronous cycling of T15 idiotype positive anti-PC PFC and anti-T15 (TEPC-15 binding) cells suggested an interaction of the idiotypic clone with anti-idiotypic regulator cells. In another system an idiotype-specific T helper cell was found to collaborate with conventional carrier-specific T helper cells to generate a T15-dominant secondary anti-PC PFC response to PC-protein antigens (77). Induction of the T15 specific T helper cell depends on the expression of naturally occurring idiotype since they were not present in mice deficient in this idiotype: (CBA/N X BALB/c)F_1 male mice expressing an X-linked defect characterized by a deficiency in T15 idiotype positive antibodies (77,78), and anti-μ suppressed mice which lack detectable levels of circulating autologous immunoglobulin (79). However, the role of T15-specific T helper cells in affecting clonal dominance is controversial since another laboratory reports that (CBA/N X BALB/c)F_1 male mice can provide adequate T cell help for a T15 dominant response (80). Although both laboratories use adoptive transfer protocols they do not

use identical T cell and B cell sources which may be responsible for the differing results.

In summary, numerous investigators have demonstrated that T15 idiotype related positive and negative regulatory components can be identified and, in some cases, can be shown to influence the PC-specific antibody response. It is possible that certain of these components may have more significant roles during the early stages of neonatal development when clonal dominance is first established, while others may function in the adult animal to maintain T15 dominance.

B cell subpopulations in the anti-PC response.

The antibody response to any given antigen involves a heterogeneous population of B cells. Analysis of the T-cell requirements for B cell stimulation with various antigens has indicated that while some B cell responses are highly dependent on T cell help, others are relatively independent of T cells (81-83) and the existence of separate B cell subpopulations having thymus-dependent (TD) and thymus-independent (TI) characteristics was proposed (81-83). TI and TD B cells have been distinguished physically on the basis of size (84) and functionally by virtue of the additivity of responses to TI and TD forms of the same hapten (10,85,86), independence in susceptibility to tolerance induction (10,81,87,88) and drug sensitivity (89,90), differences in ontogeny (91,92), and in the memory response by independent killing by BUdR and light treatment (9).

Evidence for the existence of TD and TI B cell subpopulations has included studies on the anti-PC response. Quintans and Cosenza examined precursors of PC-specific antibody forming cells in an

in vitro limiting dilution assay using R36A, a TI PC antigen (59), and PC-KLH, a TD PC antigen (85). They found that the responses to these two antigens were additive indicating that R36A and PC-KLH stimulated different B cell precursors (85). Fung and Kohler repeated the precursor analysis using the splenic fragment culture technique and also found that thymus-dependent and thymus-independent PC antigens stimulated separate precursors (93). However, in this latter study the combination of TD and TI antigens generated a superadditive response, suggesting to them that a third subpopulation responded to the combined TI + TD signal(s). However, the authors did not consider the possibility that the combination of signals could have acted to recruit additional precursors from an immature pool of cells into either or both TI and TD responsive pools. Quintans and Cosenza did not report a synergism of precursors, but noted that synergism could be observed in standard cultures where B cells were not limiting (85). Both groups suggested that there may be some overlap when TI and TD signals are given together which could indicate that these subpopulations share a common lineage at some stage of development. Expression of the T15 idiotype was also examined in these experiments, and in both studies the T15 idiotype dominated the response to both TD and TI PC antigens, suggesting that TD and TI precursors in unprimed animals arise from a common pool of B cells expressing a similar array of variable regions.

A conflicting report was published by Hurwitz *et al.* who also used the splenic fragment assay but failed to see additivity at the precursor level for TD and TI PC antigens (94). The reasons for these conflicting results are not clear. Technical differences between the

two studies were that Fung and Kohler used fetal calf serum and the isolated R36A polysaccharide while Hurwitz et al. used gamma-globulin freed horse serum and the whole R36A bacterial vaccine. However, Hurwitz et al. suggested that an important variable which cannot be easily controlled is the natural priming of different groups of B cell donors by environmental PC antigens which may influence the extent of overlapping B cell subsets.

TI and TD anti-PC B cell subpopulations have also been distinguished on the basis of susceptibility to tolerization. When adult B cells were preexposed to tolerogenic doses of PC-rabbit IgG, R36A polysaccharide or A/He anti-T15 idiotype their response to the TI antigen R36A polysaccharide was found to be much more sensitive to inhibition by all pretreatments than was the TD response to PC-hemocyanin (93). In contrast, neonatal anti-PC TD precursors were as sensitive as TI precursors to tolerogens, indicating that adult TD precursors may represent a more mature B cell subpopulation than adult TI precursors (93). These results are in agreement with studies in the DNP/TNP antigen system (87,88), where it was found that while both TD and TI neonatal TNP-specific responses were easily tolerized, adult TI precursors were much more sensitive to tolerization than adult TD precursors. However, the extreme tolerizability of TI B cells from adult mice has been challenged by Nossal and Pike who were unable to tolerize the response of adult spleen cells to DNP-polymerized flagellin (DNP-POL), another TI antigen (95). These results may be reconciled if DNP-POL is shown to be a TI-1 antigen, which is thought to stimulate a different B cell subpopulation (one which overlaps with the

TD B subset) than the TI-2 antigens such as DNP-Ficoll and R36A used in the other studies (TI-1 and TI-2 antigens to be discussed below) (96,97).

PC-specific B cells responsive to various antigen forms also appear to differ in ontogeny. Splenic PFC responses to PC-KLH and R36A polysaccharide were first detected on day 5 after birth while PC-LPS elicited a splenic response as early as 1 day after birth (55). Based on these differences, TI PC-LPS and R36A responding B cells were designated as TI-1 and TI-2 antigens, respectively. A similar subclassification of TI antigens had been proposed earlier for TI TNP antigens because of analogous differences in ontogeny (96,97), additivity of precursors by limiting dilution analysis (86), and their ability to stimulate immunodefective CBA/N B cells (96). However, this classification scheme must be interpreted cautiously since it appears that the apparent onset of responsiveness to certain TI antigens, specifically TNP conjugates of Brucella abortus, can be affected by the degree of haptenation as well as the nature of the carrier (98).

Distinct immunoglobulin isotype patterns have been noted upon immunization with different PC antigens (99). The TI antigen R36A elicits primarily IgM and IgG3 while the TD form induces primarily IgG1, lesser amounts of IgG2 and IgG3, and some IgM (99). Based on these observations, these investigators proposed that different antigen forms may stimulate separate B cell subpopulations restricted to the expression of specific isotypes, or that functional B cell subsets may represent distinct stages along a common differentiation pathway with each stage generating different isotypes. In the first case isotype

commitment would be intrinsic to a particular B cell while in the second case isotype would be determined by antigen and relevant accessory cells. Superimposed on antigen:isotype associations are preferential idiotype:isotype associations observed in the DNP/TNP response (100) and in our own studies, suggesting that V regions and C regions also may not be randomly expressed.

During their analyses of TI and TD responsive anti-PC B cell clones, Hurwitz et al. evaluated the isotype pattern elicited by each antigen type individually and by both antigen types combined (94). Their results for individual TI and TD responses confirmed those of Slack et al., and the combination of TI + TD antigens gave responses for all isotypes. Moreover, they detected a proportion of clones which produced both IgG2 and IgG3 in cultures receiving both antigens, a category of double-producers never observed when cultures were stimulated with either antigen alone. Thus, the signals delivered by TI and TD antigens appear to be additive on the basis of isotype induction, however, the non-additive precursor frequencies obtained in this study would seem to indicate that overlapping B cell subsets respond to these distinct antigen signals. The CBA/N mouse is another useful experimental model for distinguishing B cell subpopulations responsive to various antigen forms. This mouse strain carries an X-linked immune defect (X^{id}) which impairs antibody production against certain thymus-independent polysaccharide antigens, designated TI-2 antigens, such as TNP-Ficoll (101), type III pneumococcal polysaccharide (102), and polyinosinic acid (103), but permits responses to TI-1 antigens such as TNP-lipopolysaccharide (96) and TNP-Brucella abortus (96). Although

CBA/N mice responded to TI-1 antigens, antibodies produced against these antigens were selectively deficient in IgG3, normally a significant component of these responses (99). CBA/N responses to some TD antigens appeared normal except for a deficiency in IgG3 (99), however, IgG responses to certain TD antigens were quantitatively deficient (104,105) while responses to other TD antigens such as (T,G)-AL (106) and group A streptococcal carbohydrate (107) were totally absent.

In addition to functional immune deficiencies, CBA/N mice also exhibit differences in cell surface antigen expression on B cells and, for certain antigens, on macrophages. These differences include a higher $\mu:\delta$ ratio than normal mice and the lack of expression of Mls, Lyb3, Lyb5, Lyb7 and IaW.39 antigens which may reflect the absence of a subpopulation of B cells with this surface phenotype (reviewed in 108). CBA/N mice additionally lack clonable B cells which can proliferate in mitogen-stimulated semisolid agar cultures (109).

Initial studies on the PC-specific antibody response in CBA/N mice and F_1 (X^{id}/Y) male mice indicated that these mice could not respond to any form of PC, including PC coupled to TD and TI-1 carriers which presented TNP in an immunogenic form to these mice (110-112). These studies measured anti-PC hemagglutination titers of primary sera and direct (IgM) splenic PFC responses. Kishimoto et al. also failed to obtain IgM and IgG anti-PC antibodies, but detected substantial primary and secondary PC-specific IgE titers which were at least partially T15-idiotype positive in CBA/N and F_1 male mice immunized with PC-KLH in alum (13). The preservation of the IgE PC-specific response implied that IgE precursors represent a distinct subpopulation of B cells from

IgM and IgG precursors which are not under the control of the X^{id} gene. In subsequent studies, other investigators succeeded in eliciting secondary antiphosphorycholine responses which were quantitatively equivalent to normal responses but differed significantly in antibody isotype and idiotype (114-116). F_1 male mice required two or more immunizations with PC-hemocyanin or PC-LPS in order to produce detectable serum levels of anti-PC antibody. Although some animals eventually produced near-normal concentrations of antibody, these antibodies differed from normal mice in the absence of T15-idiotype positive and IgG3 components. One group was able to detect the T15 idiotype in a few animals, but in their study there was a possibility of contamination of their CBA/N colony with wild type animals (116). In addition to PC-inhibitable antibodies, F_1 male mice produced variable amounts of antibodies which were not inhibited by PC hapten and which are probably specific for the diazophenylphosphorylcholine moiety (115,116,117). It should also be mentioned that no one has yet obtained an anti-PC response in X^{id} mice immunized with the TI-2 antigen R36A, therefore this response may be missing in the secondary pool of these mice as well. Recently, Golding et al. have restored anti-TNP TI-2 responsiveness in X^{id} mice by exposing them to allogeneic (T cell) signals along along with antigen (118). In this study IgG3 antibodies were enhanced, and thus it may be possible to correct the T15 idiotype/PC-specific defect by similar mechanisms.

If all of the defects of the CBA/N mouse were parsimoniously ascribed to the absence of a single B cell subpopulation reflecting a single gene defect, this model would indicate that normal mice utilize

a distinct B cell subset to respond to PC antigens; this subset would have a surface phenotype reflecting its unique triggering mechanisms and would be committed to T15 and IgG3 expression. It is possible that this putative subpopulation could subsequently switch to the expression of other immunoglobulin isotypes (e.g., IgG1, IgA), however, the initial switch from IgM to IgG3 might be obligatory and restricted to the T15:IgG3 combination. However, the complexity of its deficiencies raises the possibility that the CBA/N mouse has multiple immune defects and that the resulting pattern of impaired responsiveness and surface phenotype may be controlled by multiple genetic loci. The X^{id} locus that precludes the response of CBA/N mice to certain TI antigens has been mapped on the X-chromosome between the genes Ta (tabby) and Hyp (hypophosphatemia) and it has not been possible to segregate the various manifestations of the X^{id} mutation (119), however, the numerous defects may result from the interaction of this X^{id} locus with other loci in other parts of the genome.

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PAPER 1.

IMMUNOLOGIC MEMORY TO PHOSPHORYLCHOLINE IN VITRO

I. Asymmetric Expression of Clonal Dominance

ABSTRACT

Anti-phosphorylcholine (PC) memory was elicited when spleen cells of primed BALB/c mice were challenged *in vitro* with PC-KLH. IgA, IgG, and IgM PFC were expressed in approximately equal proportions. Optimal responses for all immunoglobulin classes occurred when cultures received 10^{-3} to 10^{-1} μg/ml PC-KLH and were assayed on days 7 to 9. The anti-PC IgG response was expressed in all 4 subclasses although to the greatest extent in IgG1. Memory precursors for all 3 classes persisted in the spleen for at least 13 mo after priming. Analysis of PFC avidity by hapten inhibition and expression of the T15 idiotype by inhibition with a rabbit anti-T15 serum indicated that the 3 immunoglobulin classes differed in heterogeneity. IgM PFC were relatively homogeneous in avidity for PC, and the IgM hapten inhibition profile resembled that of TEPC 15. The IgA response also included PFC that were similar in avidity to TEPC 15, but in addition expressed a population of higher avidity PFC (inhibited by 10^{-6} M PC), which was undetected in the IgM response. Both IgM and IgA anti-PC PFC were dominated by the T15 idiotype whether antigen challenge occurred *in vitro* or *in vivo*. In contrast the avidity of IgG anti-PC PFC differed greatly from TEPC 15, consisting of PFC that were higher in avidity (inhibited by $\leq 10^{-6}$ M PC) and PFC that were lower in avidity (inhibited by $>10^{-3}$ M PC). Furthermore, neither *in vitro* nor *in vivo* generated IgG PFC were dominated by the T15 idiotype although expression of $T15^{+}$ IgG PFC was greater *in vivo* than *in vitro*. This asymmetry in idiotype dominance may be due to a greater proportion of T15 idiotype negative clones among IgG precursors as compared to IgM and IgA, with the

expression of these precursors being favored *in vitro*. These results suggest that 1) there may be a less rigorous maintenance of idiotype dominance among IgG memory precursors as compared to IgM and IgA memory precursors, or 2) idiotype by T15 memory precursors of the 3 isotypes may be affected by separate mechanisms.

INTRODUCTION

Mice immunized to haptens such as 2,4-dinitrophenyl (DNP) or 4-hydroxy-3, iodo-5-nitrophenacetyl (NIP) express a diverse repertoire of hapten-specific clonotypes (1-3) with immunologic memory to these determinants found in various immunoglobulin classes (4-7). In contrast, the antibody response to phosphorylcholine (PC) is typically restricted in heterogeneity (8-11), and it has been difficult to prime mice to produce anti-PC antibody of isotypes other than IgM (11) although in some cases IgG anti-PC responses have been generated *in vivo* (12-14). Depending on the immunogen, several subclasses of anti-PC IgG were represented. For example, immunization with the pneumococcus R36A elicited IgG3 antibody exclusively (12) whereas immunization with PC-Keyhole limpet hemocyanin (KLH) resulted in the production of IgG1, IgG2, and IgG3 anti-PC antibody (12-14).

In BALB/c mice serum IgG and IgM antibody and PFC responses to PC are dominated by the TEPC 15 (T15) idiotype (13,15) despite the observation that the potential repertoire of BALB/c mice at the precursor level is more diverse (16-19). Clonal dominance by T15 is probably the result of complex regulatory mechanisms that influence the differentiation of PC-specific precursors; however, it is not yet clear whether

such regulatory mechanisms affect all isotypes of anti-PC antibody equally.

In this report we describe the elicitation of anti-PC memory *in vitro*. Memory was expressed as IgG, IgA, and IgM anti-PC plaque-forming cells, many of which did not possess the T15 idiotype. Dominance of the memory response by T15 varied among the 3 immunoglobulin classes and followed the order IgM > IgA >> IgG. Inhibition of plaque formation by free hapten further indicated that the IgG and IgA anti-PC memory responses were more heterogeneous than the IgM response.

MATERIALS AND METHODS

Animals. Adult female BALB/c mice were obtained from Fred Hutchinson Cancer Research Laboratories, Seattle, WA, and were provided with food and water *ad libitum*.

Immunization. Particulate PC-KLH, was prepared by coating onto bentonite (Fisher Scientific, Fair Lawn, NJ) as described by Gallily and Garvey (20) with modifications (21). Mice were given an i.p. injection of 100 μg protein PC-KLH bentonite in 0.5 ml saline at 2-wk intervals for a total of 3 injections. They were then rested for various lengths of time before *in vitro* challenge with soluble PC-KLH as indicated in the text. Mice were primed with TNP-KLH-bentonite as previously described (22), or with sheep red blood cells (SRBC) as described in RESULTS.

Antigens. p-Nitrophenyl phosphorylcholine (Sigma Chemical Co., St. Louis, MO) was reduced and diazotized according to Chesebro and Metzger (23). The diazo-phenyl phosphorylcholine (DPPC) was used to

produce 2 preparations of PC-KLH. PC_{95} KLH was prepared by incubating 80 mg DPPC and 100 mg KLH in 50 ml 0.28 M cacodylate buffer at pH 8 for 30 min at room temperature. The solution was neutralized with 1 N HCl, incubated at room temp for 2 hr and overnight at 4°C. The protein was dialyzed for 48 hr against several changes of 0.028 M cacodylate-buffered saline, pH 7 (Cac-saline), to remove unbound hapten.

PC_{1356} KLH was prepared by reacting 69 mg KLH with 96 mg DPPC in 21 ml borate-buffered saline (0.035 M NaB_4O_7, 0.08 M NaCl) at pH 9.2 for 2 hr at room temperature. The solution was neutralized with 1 N HCl, dialyzed against cac-saline for 48 hr, and twice passed over a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) equilibrated with cac-saline to remove unbound hapten. PC_{12}-BSA was prepared by reacting 40 mg DPPC and 20.9 mg BSA in 8.4 ml borate-buffered saline, pH 9.2, for 2 hr at room temperature. The protein was dialized for 48 hr with several changes of phosphate-buffered saline, pH 7. Protein concentration was measured by Nesslerization (24) and the hapten to protein molar ratios were calculated from absorption at 475 nm in 0.1 N NaOH (25) by assuming KLH to have a m.w. of 8×10^6 and BSA a m.w. of 7×10^4.

Cell cultures. Spleen cells were cultured as described previously (25,26) in medium supplemented with 5×10^{-5} M 2-mercaptoethanol. Each well contained 10^6 cells and the contents of 4 or 8 such wells were pooled and assayed for PFC as 1 culture. At least 3 such cultures were assayed per experimental point to obtain data for calculation of the mean and standard error of the mean. At least 3 mice were used per experimental group.

Assay of in vitro plaque-forming cell (PFC) response. SRBC were conjugated with DPPC and plaqued according to the method of Claflin et al. (27). Trinitrophenyl (TNP)-SRBC were prepared according to the method of Rittenberg and Pratt (21). Cells secreting anti-PC, anti-TNP, or anti-SRBC antibody were detected by plaque assay with the appropriate SRBC and the slide technique of Cunningham and Szenberg (28). IgM PFC were detected by direct plaquing with SRBC. IgG PFC were detected by facilitation with goat anti-mouse IgG antiserum or with rabbit subclass-specific antisera (Bionetics, Kensington, MD) (26). IgA PFC were detected with rabbit anti-MOPC 315 (α, λ_2), which had been absorbed with normal SRBC, PC-SRBC, and by passage through MOPC 195 (γ, κ) and MOPC 104E (μ, λ) Sepharose 4B immunoabsorbent columns. Assays for IgG or IgA PFC contained suppressive amounts of anti-IgM antibody to inhibit direct PFC (26,29).

Inhibition of plaque formation was carried out by adding the inhibitor to the plaquing mixture during the assay. Phosphorylcholine chloride (Sigma Chemical Co.) was dissolved in 0.01 M phosphate-buffered saline, pH 7.4, and the calcium phosphate precipitate was removed by centrifugation before use for hapten inhibition experiments. Dilutions of inhibitor were made in MEM and adjusted to pH 7 when necessary. PC-BSA, purified MOPC 315 protein, pooled mouse IgG (Miles Laboratories, Inc., Kankakee, IL) or TK-1, an anti-TNP monoclonal protein (γ_1, κ) produced in our laboratory by P. Foiles, were added to the plaquing mixture as indicated in RESULTS.

The proportion of $T15^+$ PFC was determined by inhibition with rabbit anti-idiotype antiserum. Anti-idiotype or normal rabbit serum

was incubated with spleen cells for 5 min at room temperature before the other components of the plaque assay were added. In these anti-idiotype experiments a combination of all 4 rabbit subclass specific antisera was used for facilitation of total IgG PFC since normal rabbit serum or anti-idiotype serum appeared to inhibit facilitation by the goat anti-IgG antiserum used in our standard assay for total IgG PFC.

Anti-idiotype antiserum. Rabbit anti-S107 (T15) antiserum was a gift of Dr. J. Quintans and was produced by immunizing rabbits in the manner described by Claffin and Davie (30). Before use here, the antiserum and a normal rabbit serum were each heat-inactivated at 56°C for 30 min and absorbed with an equal volume of washed SRBC for 15 min at 4°C. These sera were further purified by passages over the following Sepharose 4B-linked immunoabsorbent columns: CBA/J normal serum (which lacks T15 idiotype), MOPC 315, and TK-1. After these treatments the anti-idiotypic serum produced a precipitin band in immunodiffusion against purified TEPC 15 protein but not against similar concentrations of purified MOPC 460D (α, κ), MOPC 315, MOPC 104E, or TK-1.

RESULTS

Dose response and kinetics of the in vitro secondary anti-PC PFC response. The dose response of spleen cells of primed mice cultured with different concentrations of PC-KLH are presented in Figure 1. Antigen doses ranging from 10^{-3} to 10^{-1} µg/ml produced optimal responses for all 3 Ig classes in most experiments. The isotype specificity of the plaques detected by facilitation with class-specific antisera is demonstrated in Table I in which it can be seen that

myeloma protein MOPC 315 (α, λ_2), hybridoma protein TK-1 (γ_1, κ), or pooled serum IgG specifically inhibited PFC of the appropriate isotype.

Figure 2 depicts a representative experiment (1 of 5) showing the kinetics of the in vitro secondary response. Although not tested here, in some experiments PC-specific PFC could be detected as early as day 5 of culture. In this experiment the response in all classes is maximal at day 9; however, in other experiments maximal response sometimes occurred on day 7. Consequently, we tested cultures on both days, although in subsequent experiments we only show results from the optimal day since the conclusions from either day were similar. The distribution of the 4 IgG subclasses in this in vitro response is shown in Table II. As in the in vitro memory response to TNP-KLH (5), IgG1 is the major IgG subclass expressed; however, it can be seen that all IgG subclasses are represented in the response of primed mice to PC-KLH. It is possible that there is some cross-reactivity among the subclass-specific antisera since the sum of the PFC detected by each of these reagents individually was greater than the total detected by the mixture of reagents.

Hapten inhibition of in vitro secondary anti-PC PFC. The antigen specificity and heterogeneity of the memory PFC response as demonstrated by inhibition of plaque formation with free PC hapten is shown in Figure 3. IgM and IgA responses were inhibited 80% or greater by 10^{-3} M PC. In 3 separate experiments consistent inhibition of the secondary IgM PFC response was obtained only with hapten concentrations of 10^{-5} M or greater. This inhibition profile is similar to that reported for primary anti-PC PFC (11,15,31). In contrast, IgA plaque

formation was inhibited to some extent (20% in this experiment and up to 50% in others) even by 10^{-6} M PC. Thus, there appears to be a fraction of higher avidity IgA PFC that was not detected in the IgM response. The sharp, homogeneous inhibition profile of the monoclonal TEPC 15 IgA PFC is shown for comparison. IgG PFC were only inhibited 51% at the highest (10^{-3} M) free hapten concentration, although 96% inhibition could be achieved by 10^{-5} M PC offered as a multideterminant conjugate on a protein carrier (Fig. 3, Inset). Since some IgG PFC (approximately 20%) could be inhibited by as little as 10^{-6} M PC whereas others (approximately 50%) could not be inhibited by even 10^{-3} M PC, it appears that at least 2 populations of anti-PC IgG memory precursors exist, both of which differ from T15 in PC-binding characteristics. Even the "low avidity" population was inhibited by 10^{-5} M multivalent PC (PC-BSA), indicating specificity for PC although this specificity may involve the bridge amino acid (32) as has been reported with anti-DNP (33). BSA alone did not inhibit anti-PC plaques and none of these free hapten concentrations affected the formation of IgA, IgM, or IfG anti-SRBC plaques by spleen cells of mice primed with SRBC (not shown).

Duration of anti-PC memory susceptible to in vitro challenge with PC-KLH. It was of interest to determine the time after priming required for maximal in vitro expression of PC memory and also to determine the persistence of memory to PC in these immunized animals. Table III shows the responses of spleen cells from PC-KLH primed mice challenged in vitro with PC-KLH at various times after priming. Mice were rested for a minimum of 2 mo to allow the primary antibody

response to subside since we have previously noted that this was necessary to obtain a vigorous IgG response from mice immunized with TNP-KLH in the same way as we used here for PC-KLH (26). At 2 mo after priming, spleen cells generated in vitro IgA and IgM responses that were similar or increased when compared to the primary anti-PC response of carrier primed cells. Maximal levels of IgM, IgG, and IgA response were reached by 5 mo after priming (the exception being animals primed with the low epitope preparation PC_{95} KLH, which based on these limited results appear to be slower in developing optimal memory), and substantial levels were maintained until at least 13 mo after priming. It is noteworthy that, in contrast to IgA in primary cultures, no IgG PFC were detected in primary cultures irrespective of the antigen dose used or the day of assay, although IgG memory was detected at the earliest time tested after priming.

In most of these experiments it was noted that all 3 major immunoglobulin classes were expressed to the same extent in the PC memory pool. This contrasts with other secondary responses, which are predominantly IgG, with much lower numbers of IgM and IgA PFC (5,6). When PC-KLH primed mice were challenged in vivo with PC-KLH in saline a similar class distribution of PFC was obtained (Table IV); thus the relative proportions of IgA, IgG, and IgM anti-PC memory cells triggered in vitro resembles in vivo expression of memory to the PC epitope under these conditions of priming.

Expression of the T15 idiotype in the anti-PC memory PFC response. The IgM and IgG PFC response of BALB/c mice to PC has been shown to be dominated by the T15 idiotype (13,15). However, hapten inhibition

suggested that this was not true of the *in vitro* memory response. To analyze clonal restriction more fully, we examined the pattern of T15 expression in all 3 immunoglobulin classes of the PC-memory response. T15 idiotype-specific rabbit antiserum was used to inhibit anti-PC plaques (Fig. 4). IgM PFC were nearly completely (93%) inhibited with a 1/50 dilution of the anti-idiotype. The IgA response was also strongly inhibited (92%) at 1/50 dilution. In contrast, *in vitro* generated IgG PFC were not significantly affected by the presence of anti-idiotype at any of the concentrations tested. Similar dilutions fo normal rabbit serum had no inhibitory effect and often enhanced the detection of IgM or IgA plaques; the reason for this latter effect is not known since the normal serum had been passed over Ig-immuno-absorbent columns. Parallel studies were performed on PFC generated by *in vivo* challenge of primed mice (Fig. 5). IgM PFC stimulated *in vivo* were completely inhibited by the idiotype-specific antiserum. Similarly, the IgA response elicited *in vivo* appeared identical to the *in vitro* response with respect to T15 expression. The greatest difference between *in vivo* and *in vitro* responses was seen for the IgG PFC, which were inhibited by 43% in the presence of a 1/25 dilution of anti-idiotype antiserum when antigen stimulation took place *in vivo* compared to lack of inhibition of *in vitro* generated IgG PFC (Fig. 4). Similar concentrations of anti-idiotype antiserum had no effect on anti-TNP PFC (not shown). Thus, it appears that *in vitro* conditions may favor the expression and/or expansion of non-T15 IgG memory precursors.

DISCUSSION

PC was originally characterized as an antigen that elicits exclusively an IgM response in mice (11). However, recently, production of IgG serum antibody to PC was reported in mice immunized with pneumococcal vaccine R36A (12), PC-KLH (13), or with PC conjugated to hemocyanin via a tripeptide spacer (18). Our own data demonstrate that substantial anti-PC IgG, IgA, and IgM PFC responses can be obtained both *in vitro* and *in vivo* with spleen cells of BALB/c mice primed with PC-KLH adsorbed onto bentonite. Inhibition of the majority of plaques with free PC or PC-BSA indicates that the response is hapten specific. The isotype specificity of the facilitating antisera for IgG and IgA plaque formation was confirmed by inhibition with purified proteins of the appropriate Ig class.

Representation of various Ig classes in murine secondary responses to DNP-KLH Or TNP-KLH differs from our results with PC-KLH in that IgM antibody was found to be a minor component of the memory anti-DNP/TNP response, which was predominantly IgG (5,6,26). In contrast, similar levels of IgM, IgG, and IgA were produced after *in vitro* or *in vivo* challenges with PC-KLH, indicating that this property of the response to PC was not the result of culture conditions and that it is likely a characteristic of the PC epitope rather than the carrier. Although IgG1 was the major subclass in the anti-PC response, significant levels of IgG2a, IgG2b, and IgG3 were also detected, as has been reported by others studying the *in vivo* IgG response to PC-KLH (12).

PC-specific memory cells for all 3 immunoglobulin classes could be detected for at least 13 mo after priming. *In vitro* induction of IgM,

IgA, and IgG memory has been achieved in conventional mice immunized i.p. with NIP-CGG (7), DNP-KLH (5), and TNP-KLH (26, and Chang and McCarthy, unpublished observations). Significant numbers of primary splenic IgA and IgM anti-PC precursors were detected in cultures from carrier-primed mice in the absence of a measurable IgG response (Table III). It has been suggested that the IgA response to PC observed in unimmunized mice may be the result of previous stimulation with environmental antigens (18,34,35). Our observation that the primary IgA response increased with time after carrier-priming (Table III) with conventionally maintained animals supports this hypothesis.

The proportion of the T15 idiotype in the memory PFC response of mice primed with PC-KLH-bentonite differed for the various immunoglobulin classes. Although IgM and IgA in vitro responses were predominantly T15 positive, this idiotype was not significantly expressed in the in vitro IgG response. In vivo challenge of these mice elicited a memory response that was very similar in idiotype expression to the in vitro response with respect to IgM and IgA. In contrast, anti-PC IgG PFC generated by in vivo challenge had a definite T15 component, although it made up no more than half of the total IgG. The reason for this difference in the expression of T15 idiotype by spleen cells stimulated in vivo as compared to in vitro is unclear. Humoral or cellular factors that favor idiotype dominance by the T15 components of the IgG response may function poorly in vitro.

The limited expression of the $T15^+$ IgG response observed here would appear to be at variance with the report of Claflin and Cubberley (13), which showed that mice immunized with PC-KLH produced an IgG

response that was mainly (>0.90) $T15^+$. We have repeated their priming protocol and examined the idiotype of the PFC raised in vivo and have found that the T15 component amounts to about half of the total anti-PC IgG PFC response (unpublished observations). A major difference between our experiments is that we have examined splenic PFC responses whereas they measured serum antibody that would have been of both splenic and extra-splenic origin.

Fundamental differences in variable region expression by IgM and IgA as compared to IgG memory PFC were also suggested by the hapten inhibition studies in which we found that approximately 25% of the IgG PFC appeared to be of higher avidity than T15 PFC (inhibited by $\leq 10^{-6}$ M PC) whereas an even greater proportion was of lower avidity (not inhibited by 10^{-3} M PC, Fig. 3). In contrast, the IgM response appeared relatively homogeneous with most of the PFC being inhibited between 10^{-5} and 10^{-4} M PC. Identical results were obtained by Claflin et al. (11) for secondary IgM PFC responses of BALB/c mice immunized with R36A pneumococci. Low concentrations of free hapten ($\leq 10^{-7}$ M) appear to have a slight (10%) augmenting effect on the IgA response in Figure 3. However, this effect was not observed regularly in our experiments and is probably within the error of the method. Furthermore, it is considerably lower than the 25 to 92% augmentation described recently by Schrater et al. (36), which they attributed to release from autoanti-idiotypic suppression.

The IgA response also appears to contain a population of higher avidity PFC capable of inhibition by 10^{-6} M PC. In several experiments this fraction of higher avidity PFC has ranged from 20 to 50% of the

total IgA PFC. This population may correspond to the fraction of in vitro IgA PFC that cannot be inhibited by the rabbit anti-T15 antiserum. The anti-idiotype antiserum used in these studies has been noted to react with another PC-binding myeloma protein (PCBMP), MOPC 511 (J.P. McKearn, personal communication), which shares extensive V_H N-terminal amino acid sequence homologies with TEPC 15 (reviewed in 37). Thus, the anti-idiotype appears to be specific for determinants unique to T15 (IdI) and for cross-reactive determinants shared by other PCBMP (IdX). Our finding that some of the secondary PFC were not inhibited by the anti-idiotype suggests that the antibody produced by these BALB/c cells may be idiotypically unrelated to either MOPC 511 or T15. Inclusion of such structurally distinct antibodies in the anti-PC library is supported by the recent generation of anti-PC hybridomas that synthesize antibody differing extensively in amino acid sequence from all PCBMP studied thus far (38). Previous studies of PCBMP and of anti-PC antibody have not reported a population of PFC that could be inhibited by 10^{-6} M PC. The expression of such higher avidity antibodies in the in vitro memory IgA and IgG responses suggests that affinity maturation and development of a heterogeneous antibody response can be achieved by appropriate immunization with a TD form of the PC epitope. It will be of interest to determine the idiotype(s) of these higher avidity PFC and their association with the 4 IgG subclasses since nonrandom association of specific variable regions with particular IgG subclasses depending on antigen structure has been proposed (12).

The expression of non-T15 idiotypes in the BALB/c mouse has also been noted by Gearhart et al. (18) who identified several minor anti-PC clones that produced different patterns of cross-reactivity with a rabbit anti-T15 and a mouse anti-T15. Higher levels of T15 idiotype negative clones can be generated by neonatally induced anti-idiotype suppression (39), sublethal irradiation of adult mice (40), or by transfer of fetal or neonatal liver cells into lethally irradiated recipients (41). Therefore, it is apparent that PC-specific B cells are diverse, and immunization for memory may make this diversity more apparent through clonal expansion and/or differentiation into memory precursors.

A similar shift away from a restricted primary pattern of responsiveness and toward one which is more heterogeneous upon repeated antigen stimulation occurs in the antibody response to 4-hydroxy-3 nitrophenacetyl (NP) in the C57BL/6 mouse (42,43). It appears from our study that the IgA response more closely resembles IgM in that idiotype dominance was maintained although some heterogeneity was detected. It remains to be determined whether further heterogeneity will develop if additional antigenic stimuli are provided. It is not clear from this present study whether IgG memory precursors differ in their susceptibility to a mechanism that maintains idiotype dominance in precursors of other isotypes or whether there are separate mechanisms affecting idiotype dominance in precursors of each isotype. It has been reported that idiotype dominance is already present at the precursor level in the IgM response (32); thus it is possible that the lack of dominance of T15 in IgG precursors reflects a separate origin for these memory

cells that is not expressed as antibody production in the primary response. Further analysis of anti-PC memory in this system may lead to a better understanding of the clonal development of immunologic memory.

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TABLE I

Specificity of facilitation of IgA and IgG anti-PC plaques

Protein Added[a]	Anti-PC PFC/Slide[b]			
	IgA	% Inhibition	IgG	% Inhibition
	229 ± 32		250 ± 13	
MOPC 315 (α, λ_2)	50 ± 14	78	223 ± 5	11
TK-1 (γ_1, κ)	264 ± 23	0	54 ± 14	78
Pooled mouse IgG	274 ± 19	0	78 ± 25	69

[a] Inhibitors were present during the plaque assay at the following concentrations: MOPC 315 - 32 µg/ml, TK - 1-9 µg/ml, pooled mouse IgG - 79 µg/ml.

[b] Spleen cells of mice immunized with PC-KLH on bentonite were challenged in vitro with 10^{-2} µg/ml PC-KLH and assayed on day 7 (IgA PFC) or day 8 (IgG PFC) of culture. Arithmetic mean of triplicate cultures ± SEM.

TABLE II

IgG subclass of anti-PC PFC from spleen cells of primed mice challenged *in vitro* with PC-KLH

Specificity of Rabbit-Facilitation Serum	Anti-PC PFC/10^6 Cells Assayed[a]
IgG1[b]	7,964 ± 321
IgG2a	4,214 ± 250
IgG2b	3,107 ± 786
IgG3	2,500 ± 286
IgG1 + IgG2a + IgG2b + IgG3	12,929 ± 1,107

[a] Spleen cells were challenged *in vitro* with 10^{-2} µg/ml PC-KLH 6 mo after the 3rd priming injection. Cultures were assayed on day 9 of culture. Results are expressed as described in legend of Table 1.

[b] Facilitation was performed with individual subclass-specific developing antisera or a mixture of all subclass specific antisera at a dilution equivalent to that of the individual sera.

TABLE III

Duration of anti-PC memory in primed BALB/c mice challenged *in vitro* with PC-KLH

Printing Antigen	Group No.[a]	Time after Immunization	Anti-PC PFC/10^6 Cells Assayed[b] IgA	IgG	IgM
PC-KLH	1	2 mo	897 ± 339	1061 ± 394	3836 ± 400
		8 mo	4937 ± 562	4418 ± 742	4410 ± 564
	2	2 mo	1715 ± 590	2078 ± 702	774 ± 486
		5 mo	5811 ± 1674	5027 ± 2902	7141 ± 1156
	3	6 mo	2593 ± 75	1049 ± 826	1463 ± 398
		13 mo	4140 ± 747	3875 ± 300	4382 ± 117
KLH		1 mo	52 ± 14	0	558 ± 126
		7 mo	568 ± 162	0	728 ± 109
		11 mo	426 ± 190	0	771 ± 237

[a] PC-KLH or KLH immunized mice were challenged *in vitro* with soluble PC-KLH at the indicated times after the final priming injection. Groups 1 and 2 were primed with PC_{1356} KLH whereas Group 3 was primed with PC_{95} KLH.

[b] Results are maximum PFC obtained at the optimal dose and day of assay for each experiment and were calculated as described in the legend of Table 1.

TABLE IV

Immunoglobulin class of anti-PC PFC from spleens of primed mice challenged *in vivo* with PC-KLH[a]

Antigen Dose	Mouse No.	Anti-PC PFC/10^6 Spleen Cells			Isotype Ratio	
		IgA	IgG	IgM	α:μ	γ:μ
100 μg	1	5180 ± 236	2559 ± 1658	5047 ± 291	1.0	0.5
	2	170 ± 23	350 ± 57	100 ± 6	1.7	3.5
	3	1080 ± 123	1627 ± 34	973 ± 30	1.1	1.7
1000 μg	4	4247 ± 120	4198 ± 1081	3013 ± 221	1.4	1.4
	5	4127 ± 282	4887 ± 608	3433 ± 224	1.2	1.4
	6	1373 ± 79	2307 ± 100	357 ± 65	3.8	6.5

[a] Mice immunized with PC-KLH on bentonite were challenged *in vivo* at 6 mo after priming with an i.p. injection of 100 μg or 1000 μg PC-KLH in saline and assayed for PFC 5 days later. Data are presented for individual mice as the mean of triplicate slides ± SEM.

Figure 1. Dose response of the *in vitro* secondary anti-PC response to PC-KLH. Spleen cells of mice immunized 5 mo previously with PC-KLH on bentonite were cultured with the indicated doses of PC-KLH and assayed on day 9 of culture for IgA (upper), IgG (center), and IgM (lower) PFC.

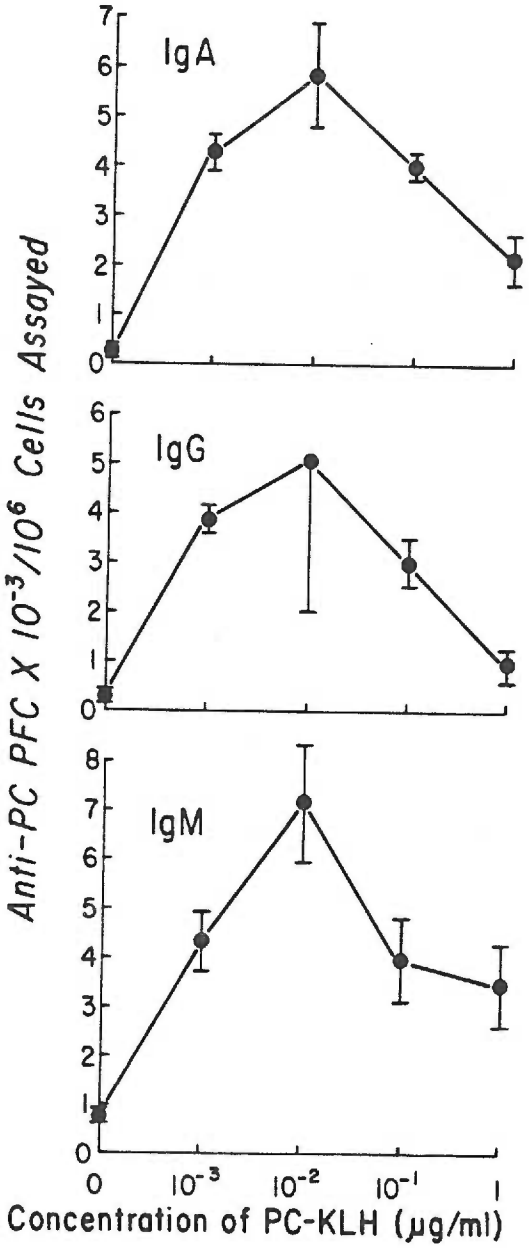

IgA
IgG
IgM
Anti-PC PFC X $10^{-3}/10^6$ Cells Assayed
0
10^{-3}
10^{-2}
10^{-1}
1
Concentration of PC-KLH (μg/ml)

Figure 2. Kinetics of the in vitro secondary PFC response to PC-KLH. Spleen cells of mice immunized 2 mo previously with PC-KLH on bentonite were cultured with 10^{-1} µg/ml PC-KLH and assayed for (x--x) IgM, (o--o) IgA, and (o--o) IgG PFC on days 6 to 9 of culture. Vertical bars indicate the SEM of triplicate cultures.

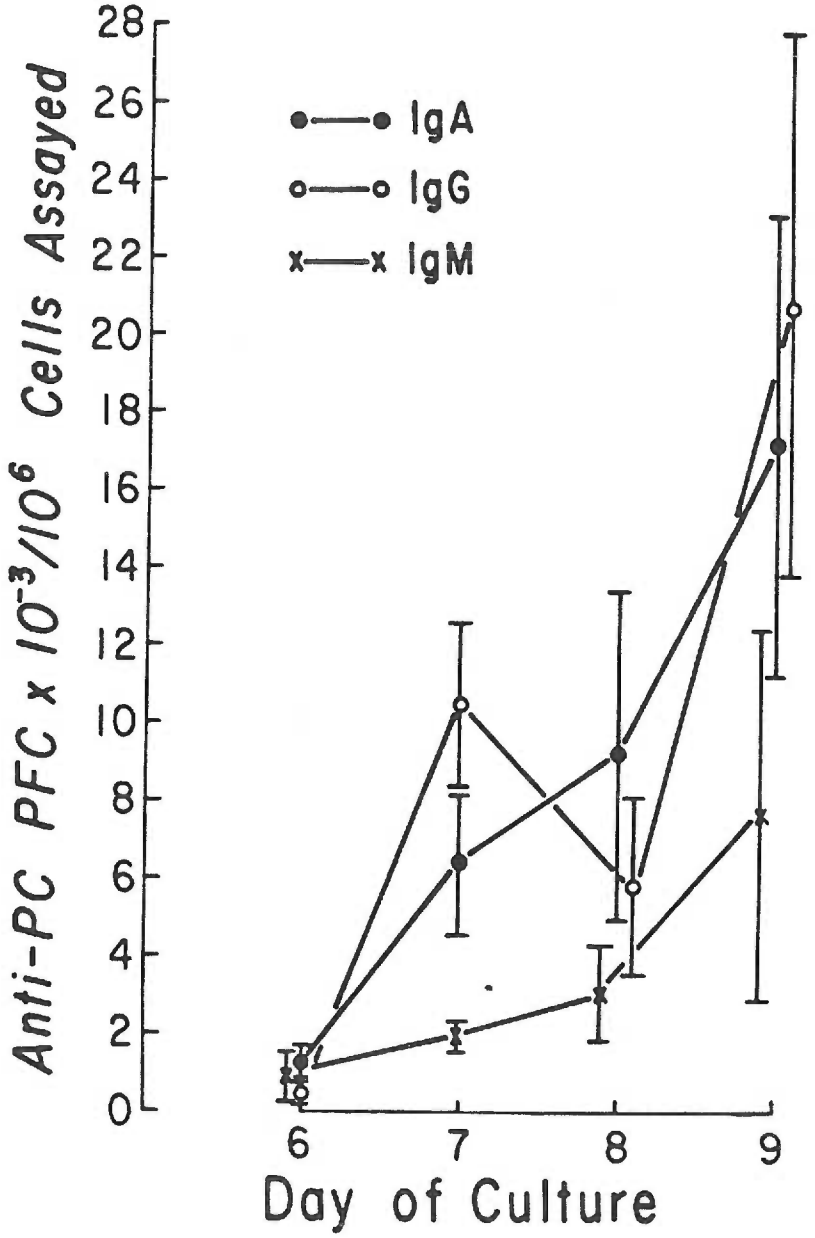

Anti-PC PFC x $10^{-3}/10^6$ Cells Assayed
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
IgA
IgG
IgM
6
7
8
9
Day of Culture

Figure 3. Inhibition of anti-PC PFC response by free hapten. Spleen cells of mice immunized with PC-KLH on bentonite were cultured with 10^{-2} µg/ml PC-KLH and assayed for PFC on day 7 (IgA, IgM) and day 8 (IgG) in the presence of the indicated concentrations of free PC, Δ--Δ IgM response, x--x IgA response, o--o IgG response, and _--_ TEPC 15 myeloma cells. Inset, Inhibition of IgG PFC by PC_{12}-BSA. BSA alone did not inhibit anti-PC IgG PFC. SRBC-specific splenic PFC of control mice given 2 injections of 5×10^8 SRBC i.p. 19 days apart and assayed 5 days after the 2nd were unaffected in the presence of the same concentrations of free PC.

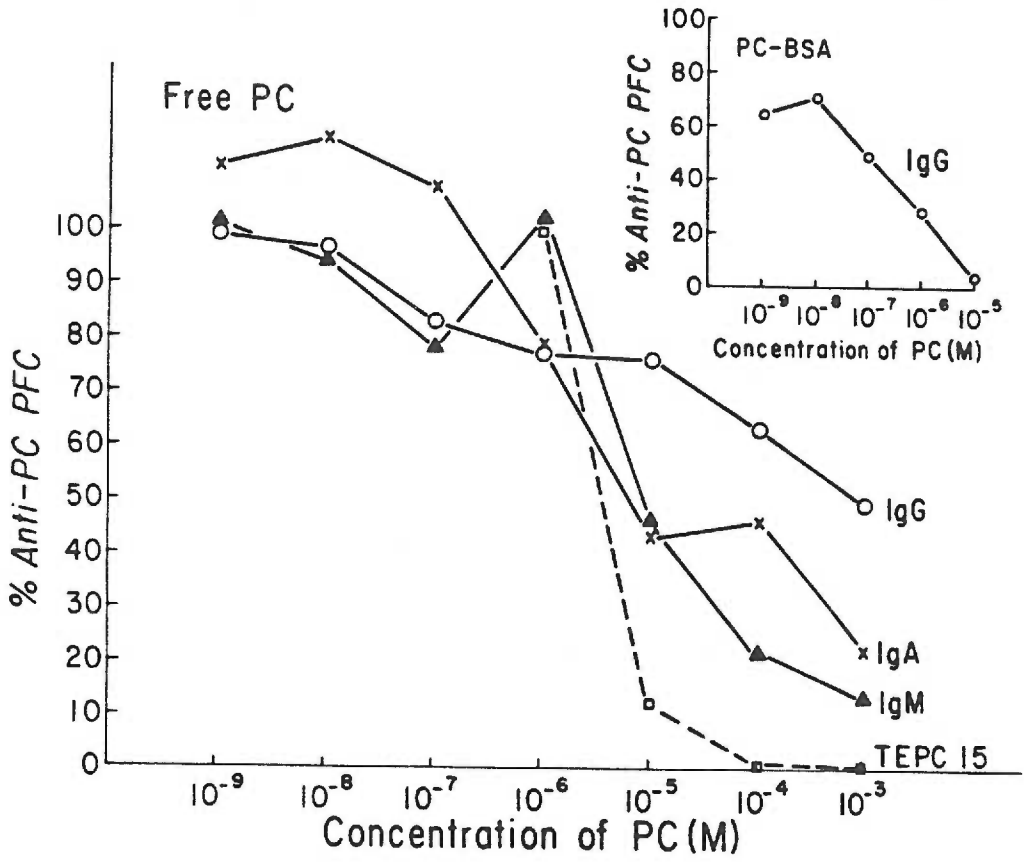

Free PC
PC-BSA
IgG
IgA
IgM
TEPC 15
% Anti-PC PFC
Concentration of PC (M)
100
90
80
70
60
50
40
30
20
10
0
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}

Figure 4. Expression of the T15 idiotype by anti-PC PFC from spleen cells of primed mice challenged *in vitro* with PC-KLH. Spleen cells of PC-KLH immunized mice were cultured with 10^{-2} µg/ml PC-KLH and assayed on day 9 for PFC. Pooled cells were assayed without added serum (open bars) or in the presence of rabbit anti-S107 (hatched bars) or normal rabbit serum (stippled bars) at the indicated dilutions. Results are presented as the mean of triplicate slides ± SEM.

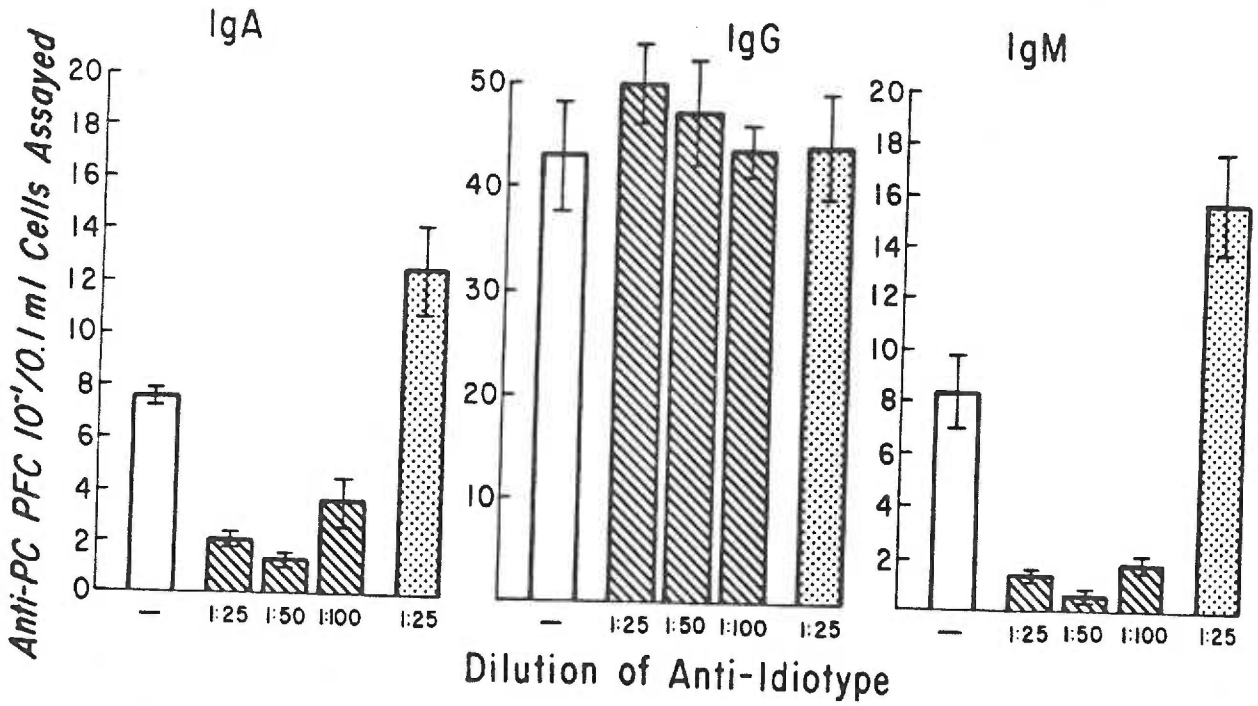
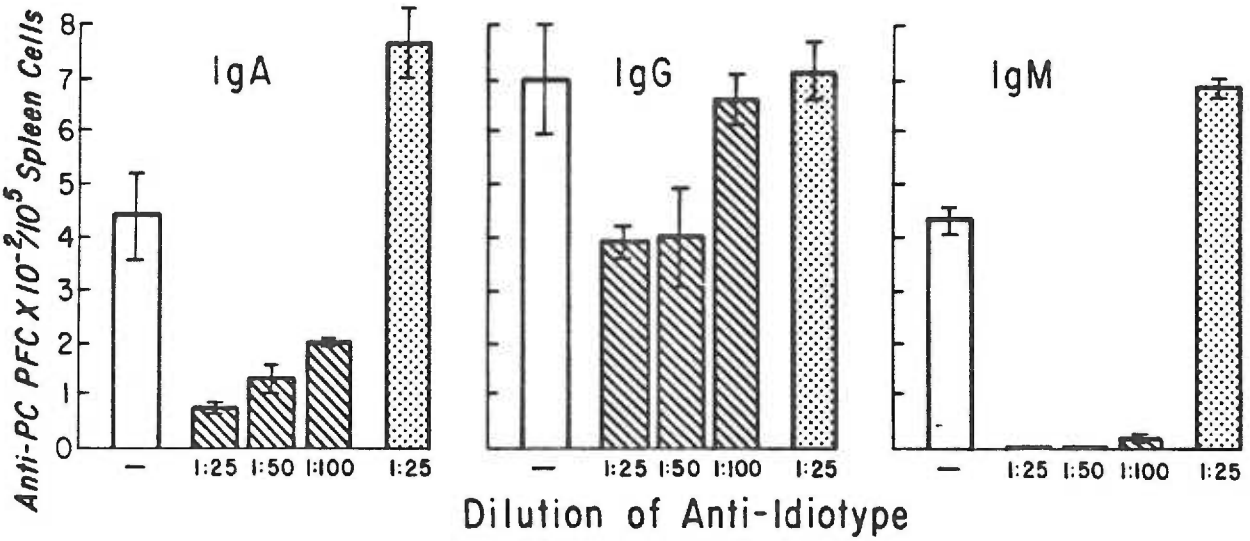

IgA
IgG
IgM
Anti-PC PFC 10^{-1}/0.1 ml Cells Assayed
0
2
4
6
8
10
12
14
16
18
20
10
20
30
40
50
—
1:25
1:50
1:100
1:25
Dilution of Anti-Idiotype

Figure 5. Expression of the T15 idiotype by anti-PC PFC from spleen cells of primed mice challenged *in vivo* with PC-KLH. PC-KLH immunized mice were challenged by an i.p. injection of 1000 μg PC-KLH and assayed 5 days later for PFC. Plaque inhibition by rabbit anti-S107 were carried out as described in the legend of Figure 4.


Anti-PC PFC X 10⁻²/10⁵ Spleen Cells
0
1
2
3
4
5
6
7
8
IgA
IgG
IgM
—
1:25
1:50
1:100
1:25
Dilution of Anti-Idiotype

PAPER 2.

IMMUNOLOGIC MEMORY TO PHOSPHORYLCHOLINE

II. PC-KLH Induces Two Antibody Populations That Dominate Different Isotypes

ABSTRACT

We examined the fine specificity of the memory response to phosphorylcholine (PC) using an enzyme-linked immunosorbent assay (ELISA) that measures the relative abilities of PC analogs to inhibit the binding of antibody to antigen. Along with the usual haptens (phosphorylcholine, L-α-glycerophosphorylcholine, and choline) these studies included as an inhibitor p-nitrophenyl phosphorylcholine, a compound that is homologous to the structure of the PC antigen in a protein conjugate. Evaluation of the fine specificity profiles revealed two populations of antibody elicited by PC-KLH, which were unequally dominant among the various immunoglobulin classes and IgG subclasses. Group I antibodies exhibited a similar or slightly greater avidity for PC than for NPPC and constituted the majority of IgM, IgA, and IgG3 antibodies. Group II antibodies, which were expressed primarily by IgG1, IgG2a, and IgG2b antibodies, possessed significant avidity only for NPPC. Possible mechanisms that would link IgM, IgA, and IgG3 to similar variable regions while excluding IgG1, IgG2a, and IgG2b from the same grouping are discussed.

INTRODUCTION

During the course of an immune response to a single determinant striking changes take place involving the shift from IgM to other antibody classes and the increase in the average antibody affinity for antigen (1). Although both phenomena depend on exposure of B cells to the cognate antigen, a direct relationship between the class-switch mechanism and the emergence of clones that produce high affinity

antibody has not been established. In the past, it has been assumed that switching generally does not alter variable (V) region expression, i.e., antibody specificity is clonally distributed (1). However, recent evidence suggests this need not be the case, and somatic variants may arise during the class switch process (2,3).

The evaluation of differences in fine specificity is a means of comparing V region expression among various antibody populations and has been used previously in the study of antiphosphorylcholine (PC) antibodies (4). In general, such studies have concluded that changes in V region expression measured by shifts in hapten specificity are not readily observed in the anti-PC response (4).

In a previous publication, however, we reported that the memory response to PC elicited by the antigen PC-KLH is characterized by the asymmetric expression of the T15 idiotype among the various immunoglobulin classes (5); IgM and IgA PFC were predominantly T15-positive, whereas the IgG response consisted of a large proportion of T15-negative PFC. In the present work, we examine the hapten specificity of IgM, IgA, and IgG antibodies of BALB/c mice primed and challenged with PC-KLH; in addition we examine the subclasses of IgG. We employ an ELISA to compare the ability of various PC analogs to inhibit the binding of anti-PC antibody. One of these analogs is p-nitrophenyl phosphorylcholine (NPPC), a compound that most closely resembles the structure of the hapten when it is conjugated to protein (6). Comparison of the specificity patterns of the various immunoglobulin classes and IgG subclasses clearly distinguishes two populations of anti-PC-KLH antibodies. The first population possesses a similar or slightly

greater avidity for PC than for NPPC and makes up the majority of IgM, IgA, and IgG3 antibodies. The second population has significant avidity only for NPPC and comprises most IgG1, IgG2a, and IgG2b antibodies. The possibilities that these two populations may be derived from dissimilar germline V genes or that the second population arises through somatic mutations occurring during immunoglobulin class switching are discussed.

MATERIALS AND METHODS

Animals. Adult, female BALB/c mice were obtained from Fred Hutchinson Center Research Laboratories, Seattle, WA, and were provided with food and water ad libitum.

Immunization. PC-KLH with a hapten to protein molar ratio of 1356 assuming KLH to have a m.w. of 8×10^6 was prepared according to Chesebro and Metzger (5,6) and coated onto bentonite (7). Mice were given an i.p. injection of 100 µg protein PC-KLH bentonite in 0.5 ml saline at 2-wk intervals for a total of three injections. They were then rested for at least 2 mo before in vivo or in vitro challenge.

Sera and culture supernatants. Primed mice were challenged with an i.p. injection of 100 µg PC-KLH in saline, and were bled 5 days later. At least three mice were used per experimental group. Spleen cells of primed mice were cultured as described (5) with an optimal dose of PC-KLH (10^{-1} to 10^{-2} µg P/ml), and supernatants were collected on day 9 of culture and pooled.

Hybridoma and myeloma proteins. Purified proteins from the myelomas MOPC 104E, RPC-5, MOPC 195, Y5606, and J606 were purchased

from Bionetics Laboratory Products, Kensington, MD. MOPC 315 protein was purified from ascitic fluid by affinity chromatography on DNP-lysine-Sepharose 4B(8). TK-1 (γ_1, κ) and TK-3 (γ_1, κ) are anti-TNP hybridomas produced in this laboratory by P. Foiles. The proteins were purified from ascitic fluid on a protein A-Sepharose 4B column (9) followed by affinity chromatography on a DNP-lysine-Sepharose 4B column. TEPC 15, MOPC 167, McPC 603, and MOPC 511 were purified from ascitic fluid by affinity chromatography on a PC Sepharose 4B column (6).

Class-specific antibodies. Anti-μ was obtained from sheep injected with MOPC 104E. Anti-α was prepared by immunizing rabbits with MOPC 315. Anti-γ3 was obtained from rabbits immunized alternately with J606 (γ_3, κ) and Y5606 (γ_3, κ). Rabbit anti-γ_1, anti-γ_{2a}, and anti-γ_{2b} were purchased from Bionetics Laboratory Products. Cross-reacting antibodies were removed from all antisera by absorption with myeloma proteins of other isotypes coupled to Sepharose 4B (10). Anti-μ was absorbed with Y5606, MOPC 315, and TK-1. Anti-α was absorbed with MOPC 104E and TK-1. Anti-γ_3 was absorbed with TK-1, TK-3, MOPC 104E, and MOPC 315. Anti-γ_1, was absorbed with MOPC 511 and Y5606. Anti-γ_{2a}, was absorbed with TK-1 and Y5606. Anti-γ_{2b} was absorbed with TK-1 and Y5606. All antisera were precipitated with ammonium sulfate and dialyzed against 0.05 M phosphate-buffered saline (PBS), pH 7.2, before conjugation to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO). The procedure of Kearney et al. (11) was followed for enzyme-antibody coupling.

Specificity of enzyme antibody conjugates. The specificity of anti-immunoglobulin class- and subclass-specific reagents was determined by a modification of the ELISA described by Engvall and Perlmann (12). Polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated overnight with purified hybridoma or myeloma proteins of each subclass (IgG1, TK-1; IgG2a, RPC-5; IgG2b, MOPC 195; IgG3, J606; IgM, MOPC 104E; IgA, TEPC 15) at 1 μg/ml in carbonate-bicarbonate buffer, pH 9.6. The plates were washed four times with PBS - 0.05% Tween 20, then four times with PBS. Varying amounts of protein of each subclass and conjugated antiserum specific for the bound protein, diluted in PBS-1% BSA, were incubated together in the coated wells for 2 hr at room temperature. The plates were washed and 0.1 ml p-nitrophenylphosphate (1 mg/ml, Sigma) in 0.9 M diethanolamine containing 1 mM $MgCl_2$, pH 9.8 was added. The enzyme reaction was stopped by the addition of 0.05 ml 2 NaOH. The absorbance at 405 nm was determined in a Titer-tek Multiskan spectrophotometer (Flow Laboratories, Rockville, MD). All assays were done in triplicate. I_{50} values were calculated as the amount of protein needed to inhibit antibody binding by 50%. Binding of alkaline phosphatase-labeled class-specific reagents to the homologous solid-phase myeloma or hybridoma proteins was inhibited at least 100-fold more efficiently by the protein of the appropriate immunoglobulin class than by proteins of other immunoglobulin classes. A direct binding assay confirmed the specificity indicated by the inhibition data.

Preparation of PC-histone. Five milligrams calf thymus histone (type II-A, Sigma Chemical Co.) were reacted with 10 mg diazophenyl

phosphorylcholine (6) in borate-buffered saline (0.035 M NaB_4O_7, 0.08 M NaCl), pH 9.0, for 2 hr at room temperature, and then were dialized against 0.05 M PBS, pH 7.0. The hapten to protein molar ratio was 3:1, calculated from the absorbance at 475 nm and assuming the average m.w. of histone to be 15,000.

Determination of fine specificity of anti-PC-KLH antibodies by ELISA. Phosphorylcholine (PC), or its analogs p-nitrophenyl phosphorylcholine (NPPC), L-α-glycerophosphorylcholine (GPC), and choline chloride (C) (Sigma Chemical Co.) were dissolved in Earle's balanced salt solution (BSS), pH 7.4. The calcium or cadmium phosphate precipitates were removed by centrifugation from PC and GPC, respectively. The haptens were diluted in BSS, pH 7.4, and were added to the appropriate dilution of serum (1/1000) or tissue culture supernatant (1/2) in BSS containing 1% BSA. A 0.1-ml aliquot of the hapten-antibody mixture was incubated for 2 hr in PC-histone-coated microtiter wells. After washing, the plates were incubated for 2 hr with the appropriate isotype-specific conjugate, and the assay was completed as described above. The I_{50} value is the concentration of hapten (mM) needed to inhibit antibody binding by 50%; I_{50} was calculated according to the method of Reed and Muench (13).

RESULTS

The TEPC 15 myeloma protein was used to determine whether the ELISA could be applied to analyze the specificity of PC-binding antibodies. The reaction between TEPC 15 and PC-histone was inhibited by each of the analogs used depending on the concentration (Fig.1).

Relative specificities calculated as the ratio of the molar concentrations of analogs that give 50% inhibition of T15 binding were: GPC/PC = 9, C/PC = 479, NPPC/PC = 9. The relative specificities for the PC analogs GPC and C determined by the ELISA agree closely with values obtained by inhibition of the precipitin reaction (14), by inhibition of the plaque assay (15), and by inhibition of a radioimmunoassay (16). Thus, it is apparent that this method may be used to examine the specificity of anti-PC antibodies.

In this study, it was relevant to evaluate the hapten analog NPPC because this structure is closely related to the chemical form of the antigen in PC-protein conjugates prepared as described by Chesebro and Metzer (6). We compared hapten inhibition by PC and NPPC of the PC-binding myeloma proteins TEPC 15, MOPC 511, McPC 603, and MOPC 167. The NPPC/PC ratios indicated distinct fine specificity patterns among the four myeloma proteins, which seem to form two subgroups; TEPC 15 and McPC 603 with NPPC/PC ratios of approximately 10, and MOPC 511 and MOPC 167 with NPPC/PC ratios close to 0.2 (Table 1).

Sera from BALB/c mice primed and boosted with PC-KLH in vivo or supernatants from spleen cell cultures challenged in vitro with PC-KLH were tested in the ELISA to determine the relative specificity of anti-PC antibodies in each immunoglobulin class and subclass. Figure 2 presents the results of a representative experiment that shows clear differences in specificity for PC and NPPC. The range of anti-PC-KLH antibody concentrations for each immunoglobulin class and IgG subclass of duplicate experiments in serum were: IgM, 90 to 120 µg/ml; IgA, 6 to 10 µg/ml; IgG1, 92 to 270 µg/ml; IgG2a, 1 to 2 µg/ml; IgG2b, 16 to

38 μg/ml; and IgG3, 82 to 410 μg/ml. Mean I_{50} values and NPPC/PC ratios for a series of experiments are compiled in Table I. IgG1, IgG2a, and IgG2b differed markedly in specificity from all four PC-binding myeloma proteins, displaying a much higher avidity for NPPC than PC with NPPC/PC ratios of <0.004. The striking exception was the IgG3 subclass, which closely resembled IgM and IgA antibodies in relative specificity for the two haptens. It appears, however, that these latter antibodies do not belong to a single myeloma protein subgroup (TEPC 15-McPC 603 or MOPC 511-MOPC 167) because their NPPC/PC ratios lie between those of the two subgroups. The most likely explanation is that memory IgM, IgA, and IgG3 pools contain representatives of both subgroups. Because a small fraction of IgM, IgA, and IgG3 antibodies are inhibited by 10^{-2} M NPPC but not by 10^{-2} M PC (Fig. 2), these antibodies probably also contain some combining sites similar to those found in IgG1, IfG2a, and IgG2b antibodies.

The specificity analysis was extended to a comparison of the fine specificity of serum IgM, IgA, and the two major IgG subclasses IgG1 and IgG3 antibodies for all four hapten analogs (Table II). Again the difference between IgG1 antibodies and IgM, IgA, and IgG3 was striking. It is noteworthy, however, that the actual I_{50} values of IgA and IgG3 were substantially lower than that of IgM, although their NPPC/PC ratios were similar (Tables I and II).

DISCUSSION

Our experiments indicate that PC-KLH elicits two groups of antibodies distinguished by their specificities for PC AND NPPC, which are

unequally expressed by the various immunoglobulin classes. Group I consists of antibodies with relatively similar avidities for PC and NPPC, and which comprise the majority of IgM, IgA, and IgG3 antibodies. These antibodies appear to be related in specificity to the prototype anti-PC antibody TEPC 15, and to other PC-binding myeloma proteins (MOPC 511, McPC 603, MOPC 167), although on the basis of their relative avidities for PC analogs these myeloma proteins do not form a homogeneous group (see Table I and Reference 4). Group II antibodies bind NPPC but not PC and are expressed by most IgG1, IgG2a, and IgG2b antibodies. These antibodies are distinct in specificity from TEPC 15 and the three other PC-binding myeloma proteins. Differences in specificity of these two groups are extended by the use of two other commonly used PC analogs, C and GPC. Group I antibodies bind GPC, and to a much lesser degree, bind C, whereas group II antibodies do not bind either hapten significantly.

Serum concentrations of the various isotypes indicate IgG1 and IgG3 antibodies are the major isotypes elicited by PC-KLH in the memory response. Because group I and group II antibodies predominate the IgG3 and IgG1 responses, respectively, it appears these specificity groups are represented equally in the overall expression of memory to PC-KLH.

These results are consistent with our previous studies on the memory response to PC-KLH, which showed that IgM and IgA PFC were dominated by the T15 idiotype, whereas the IgG response was largely non-T15 (15). In these earlier experiments the IgG subclasses were not studied individually. Together the analyses of idiotype and binding specificity suggest the V regions possessed by most of the IgM, IgA,

and probably IgG3 antibodies are part of the T15 family whose members reportedly express the product of a single V_H germline gene in association with a V_K-22 light chain (17). The fine specificity data also suggest that a smaller but significant proportion of IgM, IgA, and IgG3 antibodies may express the T15-defined V_H gene in association with V_K-24 and V_K-8 light chains, similar to McPC 603, MOPC 511, and MOPC 167 (18). The V regions expressed by the majority of the IgG1, IgG2a, and IgG2b population as a whole are very different and could represent the product of other germline gene(s). The latter could represent distinct pairs of V_L and V_H genes not defined in previous assessments of the anti-PC repertoire or a difference in either V_L or V_H genes. These V genes need only be expressed by a minor population of B cells, which are selected and expanded by the PC-KLH priming regimen. The existence of such minor clones in the anti-PC repertoire has been reported (19). Furthermore the existence of a new V_H group unrelated to any previously defined V_H groups was suggested recently with the finding of a PC-binding IgM hybridoma from T15-suppressed mice (20). Nonrandom association of V regions with particular IgG subclasses has been postulated in the murine response to bacterial carbohydrates, and a subclass-specific mechanism operating at the level of antibody-forming cell precursors was proposed (21).

An alternative hypothesis to explain the appearance of two specificity groups in the memory response to PC-KLH would be that all memory antibody-producing cells are derived from a common family of group I-like clones by somatic mutation. Group II clones would represent an expanded population of somatic variants arising during class-switching

as suggested by Gearhart et al. (2). This model predicts that group II antibodies would be absent in the primary IgM response, an hypothesis we are currently testing.

It must be emphasized that these studies do not indicate a total absence of T15-related variable regions in IgG1, IgG2a, and IgG2b. The ability of T15-positive IgM-producing B cells to give rise to IgA and IgG1 progeny expressing the parental variable regions is well documented (22). Also, serum IgG1 and IgG2 antibodies bearing the T15 idiotype have been detected in PC-KLH immunized mice by others, although the proportion of the entire IgG1 or IgG2 responses these represented was not given (23). These observations are compatible with either of the two hypotheses discussed above because a small number of clones in the memory response may express V regions switched intact from T15-positive primary IgM to secondary IgG of the various subclasses.

We noted previously (5) that the IgG and IgA memory PFC responses contained a component with high avidity for PC, suggesting that affinity maturation was occurring within these classes. Comparison by Student's t-test of PC I_{50} values of memory IgM antibodies with those of the other classes (Table I, line 4) indicates a significant increase ($p < 0.01$) in avidity of IgA and IgG3 for the PC hapten (12 to 47-fold). These differences in avidity imply that although IgM, IgA, and IgG3 antibodies are derived from the T15 family, their binding sites are not identical. The observed increases among IgA and IgG3 group I antibodies may actually be underestimates because there are most likely some group II antibodies present in the IgA and IgG3 memory

populations. The designation of the IgM antibody in this analysis as memory antibody must be considered arbitrary; we have found no significant difference in PC I_{50} values between primary and secondary IgM (data not shown). All of the data presented here were obtained with the BALB/c mouse, and we are presently examining other strains for the expression of group I and group II antibodies. Of particular interest are strains whose primary responses are not dominated by the T15 idiotype, such as C57BL/6 (24). Our preliminary results indicate that although group I and group II are both expressed in this strain, there are subtle differences from BALB/c in the association of these antibody populations with the various isotypes (not shown).

It is possible that group II antibodies are related to the anti-"bridge" antibodies described by others (25), although the actual specificity of these latter antibodies has never been characterized. Group II antibodies did not bind p-nitrophenylphosphate (not shown), suggesting it is the PC moiety of NPPC rather than the region closer to the protein carrier that plays the more dominant role in the structure recognized by these antibodies. The pneumococcus R36A provides a naturally occurring form of PC and gives rise to antibody that is readily inhibited by free PC (4). Thus it is striking that group I IgM, IgA, and IgG3 antibodies, which are also specific for the free PC determinant, are elicited through a diazophenyl-bound PC that provides a different structural context for the PC hapten than the C polysaccharide of the pneumococcus.

A similar shift in specificity with priming is seen in the anti-NP (4-hydroxy-3-nitrophenacetyl) response of C57BL/6, in which a largely

heteroclitic (NIP-specific) primary response becomes more heterogeneous upon hyperimmunization, and involves antibodies with a higher affinity for the homologous hapten, NP (26). It is important to recognize that memory to PC-KLH is a complex response, a significant portion of which consists of heteroclitic anti-PC antibody confined mainly to IgM, IgA, and IgG3, whereas the majority of the IgG1, IgG2a, and IgG2b antibodies must be classified as homoclitic, because they bind the immunizing form of the hapten (NPPC) more strongly.

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TABLE I

Binding specificity of myeloma proteins and anti-PC-KLH antibodies for PC and NPPC

	TEPC 15	MOPC 511	McPC 603	MOPC 167		
PC	0.011[a]	0.170	0.018	0.187		
NPPC	0.102[a]	0.043	0.170	0.036		
NPPC/PC	9.5[b]	0.25	9.5	0.20		
	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
PC	>10[c]	>10	>10	0.0075 ± 0.0015	0.35 ± 0.14	0.029 ± 0.006
NPPC	0.037 ± 0.005[c]	0.022 ± 0.005	0.044 ± 0.017	0.017 ± 0.005	0.91 ± 0.31	0.061 ± 0.019
NPPC/PC	<0.004[c]	<0.002	<0.004	2.4 ± 0.6	3.6 ± 0.9	2.9 ± 1.0

[a] Concentration of PC or NPPC (mM) required to inhibit by 50% (I_{50}) binding of the myeloma proteins and antibodies to PC-histone.

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

[c] Mean ± SE of the mean for I_{50} values with each hapten and NPPC/PC ratios for each experiment. Number of different experiments done with both haptens was: IgG1, 5; IgG2a, 3; IgG2b, 3; IgG3, 7; IgM, 5; IgA, 9. Each experiment was done in triplicate.

TABLE II

Binding specificity of serum anti-PC-KLH antibodies for PC, NPPC, GPC, and C

	I_{50} (mM)[a]			
	NPPC	PC	GPC	C
IgM	1.0	0.79	2.51	>10
IgA	0.050	0.050	0.050	5.01
IgG3	0.020	0.010	0.025	1.26
IgG1	0.040	>10	>10	>10

[a] As in Table I.

Figure 1. Ability of PC analogs to inhibit binding of TEPC 15 protein to PC-histone-coated plates. Various concentrations of hapten (indicated on the abscissa) were mixed with TEPC-15 protein (0.1 µg/ml) and tested for binding in the ELISA as described in Materials and Methods. PC (o--o); NPPC (o--o); GPC (Δ--Δ); C chloride, (_--_).

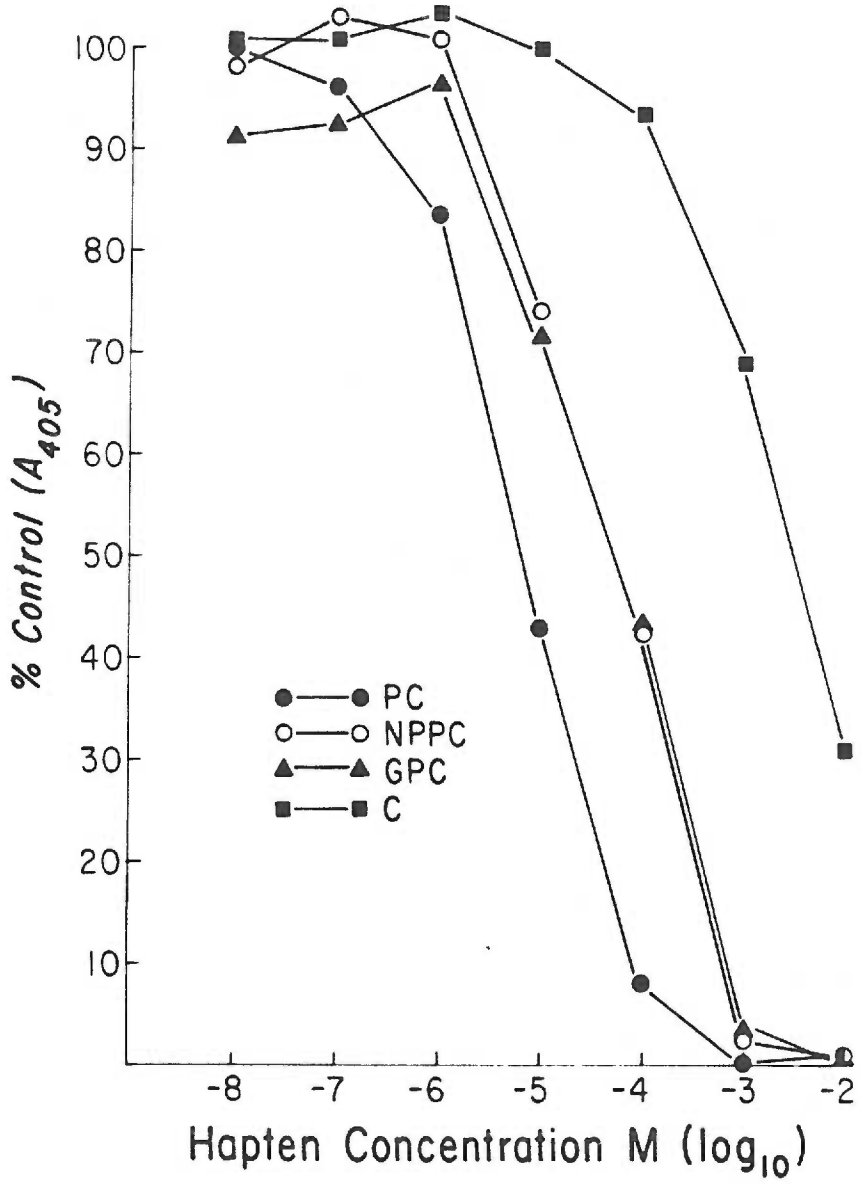
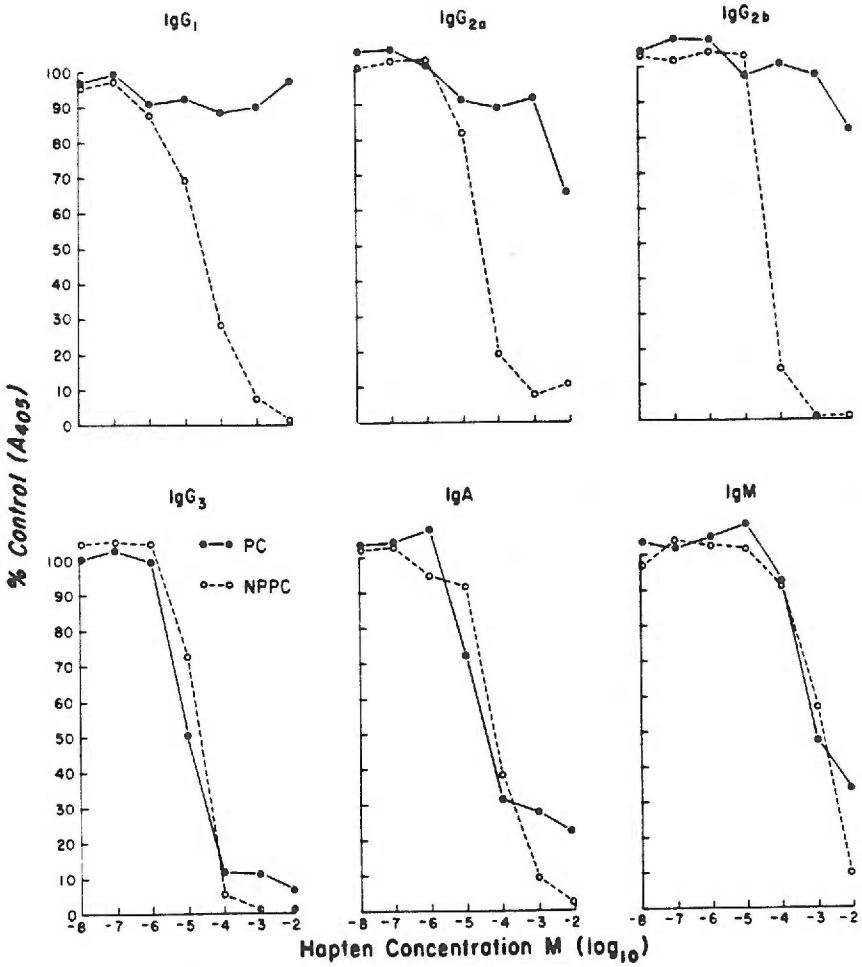

100
90
80
70
60
50
40
30
20
10
% Control (A_{405})
PC
NPPC
GPC
C
-8
-7
-6
-5
-4
-3
-2
Hapten Concentration M (log_{10})

Figure 2. Binding specificity of anti-PC-KLH immune serum. Inhibition of binding to PC-histone by PC (o--o); NPPC (o--o) was determined by the ELISA as in Figure 1.


IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
PC
NPPC
% Control (A405)
Hapten Concentration M (log10)

PAPER 3.

IMMUNOLOGIC MEMORY TO PHOSPHORYLCHOLINE

III. IgM Includes A Fine Specificity Population Distinct From TEPC 15

ABSTRACT

Group I and Group II variable regions expressed in the PC-KLH memory response may originate from distinct germline genes or Group II may arise by somatic mutation of Group I (TEPC 15) germline genes. A current version of the somatic mutation hypothesis proposes that somatic mutation is activated by the class switch from IgM to IgG or IgA. If Group II results from somatic mutation during class switching, Group II IgM antibodies would not exist. This prediction was tested in the present experiments. Group I and Group II antibodies were separated from whole serum by affinity chromatography on PC-Sepharose. An ELISA was used to characterize the fine specificity and idiotype of the isolated antibody populations. Group I antibodies were inhibited by both PC and NPPC haptens and were T15 idiotype positive. Group II antibodies were inhibited appreciably only by NPPC and were negative for the T15 idiotype. The purified Group II antibodies contained a significant IgM component and high levels of Group II IgM were detected during the early secondary response to PC-KLH. These observations are inconsistent with the hypothesis that Group II originates by somatic mutation activated by the class switch. These results strongly suggest that one or more of the germline genes (V, D or J) of Group I and Group II are different. Alternatively, if Group II antibodies arise from Group I by somatic mutation, this mutation must occur prior to class switching.

INTRODUCTION

The memory response to PC-KLH is a complex phenomenon, involving two antibody populations distinct in fine specificity and idiotype (1,2). Group I antibodies correspond in fine specificity to TEPC 15 and comprise the majority of memory IgM, IgA and IgG3 antibodies. Group II antibodies display a fine specificity pattern distinct from Group I antibodies and TEPC 15 and are found primarily among memory IgG1, IgG2a and IgG2b antibodies.

It has been suggested by others that antibodies specific for phosphorylcholine (PC) are encoded by a limited number of V_H and V_K genes corresponding to the T15 gene family which undergo somatic diversification upon class switching to generate a large number of unique variable regions (3,4). Consequently, a basic question to be asked of the memory response to PC is whether Group II antibodies represent somatic variants derived from the T15 family or whether these antibodies represent the products of one or more different germline genes. If Group II antibodies are generated by somatic mutation occurring during class switching Group II IgM antibodies should not exist. We have tested this hypothesis and find that Group II IgM antibodies can be isolated from serum of PC-KLH immunized BALB/c mice, and can constitute a major component of the IgM response early in the secondary immune response to this antigen. Furthermore, we were unable to detect the T15 idiotype in isolated Group II antibodies although the idiotype was clearly expressed by the Group I population. These results indicate that Group II antibodies arise prior to class switching; either they are the products of germline gene(s) distinct from the

T15 family or they reflect somatic mutations which occurred prior to switching.

MATERIALS AND METHODS

Animals. Adult, female BALB/c mice were obtained from Fred Hutchinson Cancer Research Laboratories, Seattle, WA and the Jackson Laboratory, Bar Harbor, ME and were provided with food and water ad libitum.

Immunization. PC-KLH with a hapten to protein molar ratio of approximately 1000 (different preparations ranged from 700-1500 assuming KLH to have a mw of 8×10^6) was prepared according to Chesebro and Metzger (5) and coated onto bentonite when priming for long term memory experiments (1). Mice used for long term memory were given an ip injection of 100 µg protein PC-KLH-bentonite in 0.5 ml saline at 2 week intervals for a total of three injections. They were then rested for at least 2 months before in vivo challenge. Primed mice were challenged with an ip injection 2 weeks apart of 100 µg protein PC-KLH in saline and were bled 5 days later. Mice used for primary and early secondary experiments were given two ip injections of 100 µg protein PC-KLH in 0.2 ml saline emulsified with an equal volume of Complete Freunds Adjuvant (CFA) and were bled at several intervals after each injection as indicated in the Results. In experiments using pooled sera at least 3 mice were used per group.

Purification of anti-PC antibodies by affinity chromatography. Anti-PC serum diluted 1:2 in 0.1 M phosphate buffer, pH 8.0, was passed over a PC-Sepharose 4B column (5). The column was washed extensively

in 0.1 M phosphate buffer, pH 8.0, then washed in 0.05 M N-carbobenzoxyglycine prior to elution with 0.01 M PC (Sigma Chemical Co., St. Louis, MO). Elution with PC was continued until no further protein could be detected in the eluate as monitored by absorbance at 280 nm, and this protein peak was designated Group I. Group II antibodies were then eluted by addition of 0.01 M p-nitrophenyl phosphorylcholine (NPPC, Sigma Chemical Co.). The collected fractions were dialyzed against PBS, pH 7.4.

Determination of binding specificity of anti-PC-KLH antibodies by the ELISA. The fine specificity of serum and purified anti-PC antibodies was determined in an ELISA as described previously (2). Briefly, PC or NPPC at different concentrations was mixed with the anti-PC antibody and incubated in PC-histone coated microtiter plates. Antibody subclass was detected by a second incubation using isotype-specific antisera conjugated to alkaline phosphatase. The enzyme-antibody conjugates showed less than 1% cross reactivity with immunoglobulins of other isotypes. The preparation and specificity of these reagents was described previously (2). The cleavage of p-nitrophenylphosphate was monitored by absorbance at 405 nm.

T15 idiotype analysis by ELISA. AB1-2 hybridoma cells producing anti-T15 (6) were obtained from the American Type Culture Collection, Rockville, MD and were grown as an ascites and the antibody purified on a protein A-Sepharose 4B Column (7). The amount of T15 idiotype present in the purified serum fractions was determined in a competition assay using the ELISA with TEPC 15 as a standard. Microtiter plates were coated with the purified anti-T15 at 3 μg/ml. After washing the

plates varying amounts of TEPC 15 protein, Group I antibody or Group II antibody were incubated together with 0.38 μg/ml alkaline phosphatase-conjugated TEPC 15 (8) for 1.5 hr at room temperature. The substrate p-nitrophenylphosphate was added to the washed plates and the O.D. determined at 405 nm.

RESULTS

Group I and Group II antibodies produced in long term memory were separated by sequential elution of serum anti-PC-KLH antibodies from a PC-Sepharose immunoabsorbent with 10^{-2} M PC (Gr. I) followed by 10^{-2} M NPPC (Gr. II). The fine specificities of the four major isotypes IgM, IgA, IgG1 and IgG3 in the two separated fractions were compared to those of unseparated serum antibodies; the results of a representative experiment are shown in Table I. The PC eluate (Gr. I) contained most of the IgM, IgA and IgG3 antibodies while the NPPC eluate (Gr. II) contained the majority of IgG1, IgG2a (not shown) and IgG2b (not shown). However, it is important to note that IgM antibodies were recovered in both Group I and Group II fractions and displayed the fine specificity typical of each group.

Fine specificity analysis of the separated fractions reinforced several observations made with whole serum antibodies. Among Group I antibodies, the differences in PC I_{50} values for the various isotypes were more evident than in unseparated serum. IgG3 antibodies in particular consistently displayed high avidity for PC. As expected, antibodies in the Group II fraction generally possessed measurable avidity only for NPPC. The basis for this specificity is not known;

however, it seems clear that the PC moiety of NPPC plays a dominant role in the structure recognized by Group II antibodies since these antibodies do not bind p-nitrophenylphosphate (2). In this experiment the IgG3 antibodies eluted by NPPC and, therefore, classified as Group II behaved somewhat like Group I as they were inhibited by 1 mM PC; however, the yield of IgG3 in this fraction was extremely low (0.4%) and contamination by Group I IgG3 antibodies trailing from the PC eluate cannot be excluded. However, the I_{50} for NPPC of these antibodies was similar to that of the other Ig classes obtained in the Group II fraction.

The separated Group I and Group II antibodies were also tested for expression of the T15 idiotype. Figure 1 presents one of four experiments that gave similar results; it shows that Group I antibodies were equivalent to TEPC 15 in ability to inhibit the binding of alkaline phosphatase-labeled TEPC 15 to the monoclonal anti-T15 AB1-2. In contrast, Group II antibodies were unable to inhibit this reaction even at the highest concentration examined which was 20 times greater than the minimum concentration of Group I that showed inhibition. Thus, Group I is clearly T15 idiotype positive while Group II is T15 idiotype negative.

In all of our previous experiments anti-PC-KLH antibodies were obtained from mice primed and rested for at least two months before challenge with antigen, a protocol which allows us to study long-term memory in resting populations (1). In order to determine when the two fine specificity groups arise during an immune response, mice were primed with PC-KLH in CFA and boosted in the same manner two weeks

later. The mice were bled at several intervals after each immunization. The fine specificities of these primary and early secondary anti-PC-KLH antibodies were analysed and compared to the profiles obtained with long term memory antibodies (2). Table II shows results for two separate pools of mouse sera and for two individual sera selected to illustrate the differences between individuals.

Early during the primary response (d7, d10) relatively high NPPC/PC ratios were often obtained (≥1) for all immunoglobulin classes, indicating an early predominance of Group I (T15-like) antibodies, an observation consistent with the findings of others (9-11). The most remarkable finding in Table II is that the early secondary and to a lesser extent the late primary IgM antibodies apparently possess a large Group II component characterized by a low NPPC/PC ratio. This contrasts with the early primary and long term memory phases during which the IgM response is predominantly Group I.

Occasionally, low NPPC/PC ratios were seen for primary IgG1 antibodies as shown for the pooled mouse sera indicating that Group II antibodies can be expressed early in the immune response in association with this IgG subclass. However, it must be emphasized that at this point during the primary response IgG1 antibodies represent less than 1% of total anti-PC antibodies and Group I TEPC-15-like antibodies are clearly predominant. Our ability to detect primary IgG anti-PC antibodies in this study contrasts with our previous observation (1) that such antibodies were not detected in the splenic plaque-forming cell assay. This difference may be due to the study of serum antibody here

using the ELISA and/or to the use of Freund's adjuvant; we previously used bentonite as adjuvant (1).

DISCUSSION

Two groups of antibodies distinguished by their specificities for PC and NPPC have been defined in the memory response to PC-KLH (2). Group I contains antibodies which closely resemble TEPC 15 and the other PC-binding myeloma proteins in fine specificity and express the T15 idiotype. These antibodies correspond to the IgM and IgG populations elicited by PC-KLH immunization of BALB/c reported by others (11,12). In contrast, Group II anti-PC-KLH antibodies are distinct from all PC-binding myeloma proteins in fine specificity and do not express the T15 idiotype. These observations raised the question of the origin of Group II antibodies: do they represent germline genes distinct from those expressed by Group I or do they arise from Group I clones by somatic mutation? One recently advanced feature of the somatic mutation theory is that the mutation mechanism is activated by the class switching event(s) (3,13). This model predicts that if Group II arises as a result of somatic mutation during or after switching Group II IgM antibodies would not be detected, an hypothesis which is tested in the present work.

Separation of Group I and Group II antibodies was achieved by sequential elution with PC followed by NPPC of antibodies bound to a PC-immunoabsorbent. This protocol effectively separated the two populations as judged by the characteristic NPPC/PC ratio of each group as well as by the expression of the T15 idiotype. A significant

although minor IgM component was recovered in the NPPC eluates which displayed the typical fine specificity pattern (low NPPC/PC ratio) of Group II antibodies. In addition, examination of the fine specificity of whole serum antibody obtained in an early secondary response often revealed a large proportion of Group II IgM antibodies not seen in whole sera of either early primary or long term memory responses. The results in Table II suggest that IgM Group II antibodies are more readily detected early after priming and that in resting memory cells most of these V region products have been switched to other isotypes in particular IgG1 but also to IgG2a and IgG2b. We have discussed elsewhere (1,2) the paradox that IgG3 antibodies continue to be dominated by the T15 idiotype and Group I characteristics even in long term memory. The demonstration here that IgG Group II antibodies must have developed from IgM Group II precursors only emphasizes the paradox since the order of the constant region genes on the chromosome would predict somewhat comparable levels of IgG3 and IgG1 Group II molecules unless there is a selective mechanism that tends to exclude $\gamma 3$ (or favor $\gamma 1$) in Group II switching.

The existence of IgM Group II antibodies is inconsistent with the hypothesis that they arise from Group I through somatic mutations correlated with the class switch from IgM to IgG or IgA and strongly suggests that Group I and Group II IgM antibodies and their clonal progeny arise from distinct germline genes. Group II may include antibodies which are distinct from the T15 family, expressing a V_H gene other than the V1 gene which encodes the T15 V_H-4 (4) region and/or V_L genes unrelated to the VK-22, VK-24 or VK-8 regions (14).

The existence of a novel V_H region, V_H-12, was demonstrated recently among the anti-PC antibodies of T15-idiotype suppressed BALB/c mice suggesting the existence of a different germline gene (15). It is also possible that the fundamental difference between Group I and Group II antibodies lies in the J_K, J_H or D_H regions utilized by the two groups. Alterations in J_H have been shown to alter the binding specificity of anti-PC antibodies (16); thus the third hypervariable region could play an important role in determining the way in which the azophenyl moiety of the PC-protein conjugate is recognized by Group I and Group II antibodies.

Alternatively somatic mutations which occur prior to the class switch such as in V-J joining could account for Group II molecules. Several investigators have analyzed germline and variant myeloma sequences and have noticed a high mutation frequency in the area of V-J rearrangement and its flanking regions, leading them to propose the activation of a localized mutation mechanism during DNA replication accompanying V-J joining (17,18).

The appearance of a large Group II IgM response only in the early secondary response suggests that these precursors are infrequent in unprimed BALB/c mice and require considerable expansion before they can be detected. The subsequent decline in the proportion of these IgM antibodies in the long term memory response may indicate that the majority of these clones have switched to IgG production. The variation between individual animals (Table II) could reflect the kinetics of the expansion and/or the switching of the Group II IgM population. The significance of these events is that a large proportion of PC-KLH

induced memory cells are derived from a very minor primary pool subject to strong selective forces during memory development apparently involving the switching of these clones to IgG1 (and to a lesser extent, IgG2b and IgG2a) production.

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TABLE I

Comparison of binding specificity of serum anti-PC-KLH antibody with Group I and Group II antibodies isolated by affinity chromatography[a]

		IgM	IgA	IgG3	IgG1
Whole Serum	PC	.87[b]	.053	.003	>10
	NPPC	.74[b]	.060	.032	.062
	NPPC/PC	.85[c]	1.1	12.3	<.006
PC-Eluate (Group I)	PC	.079	.028	.004	.007
	NPPC	.50	.25	.025	.016
	% Eluted Antibody	88	75	99.6	19
	NPPC/PC	6.3	9.0	6.3	2.3
NPPC Eluate (Group II)	PC	>10	>10	1.0	>10
	NPPC	.014	.025	.040	.032
	% Eluted Antibody	12	25	0.4	81
	NPPC/PC	<.0001	<.003	.04	<.003

[a] Approximately 8 mg anti-PC antibody was applied to each PC-Sepharose column. The recovery of anti-PC as Group I (PC-eluate) and Group II (NPPC eluate) antibodies combined was approximately 75%. The amount of antibody recovered for each immunoglobulin class was: IgM - 0.07 mg, IgA - 0.05 mg, IgG3 - 4.8 mg, IgG1 - 1.1 mg, IgG2a - .0008 mg, IgG2b - 0.002 mg.

[b] I_{50} values represent the concentration of PC or NPPC (mM) required to inhibit binding of antibody to PC-histone by 50%.

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

Table II

Binding specificity of primary, early secondary and long term memory anti-PC-KLH antibodies in BALB/c mice

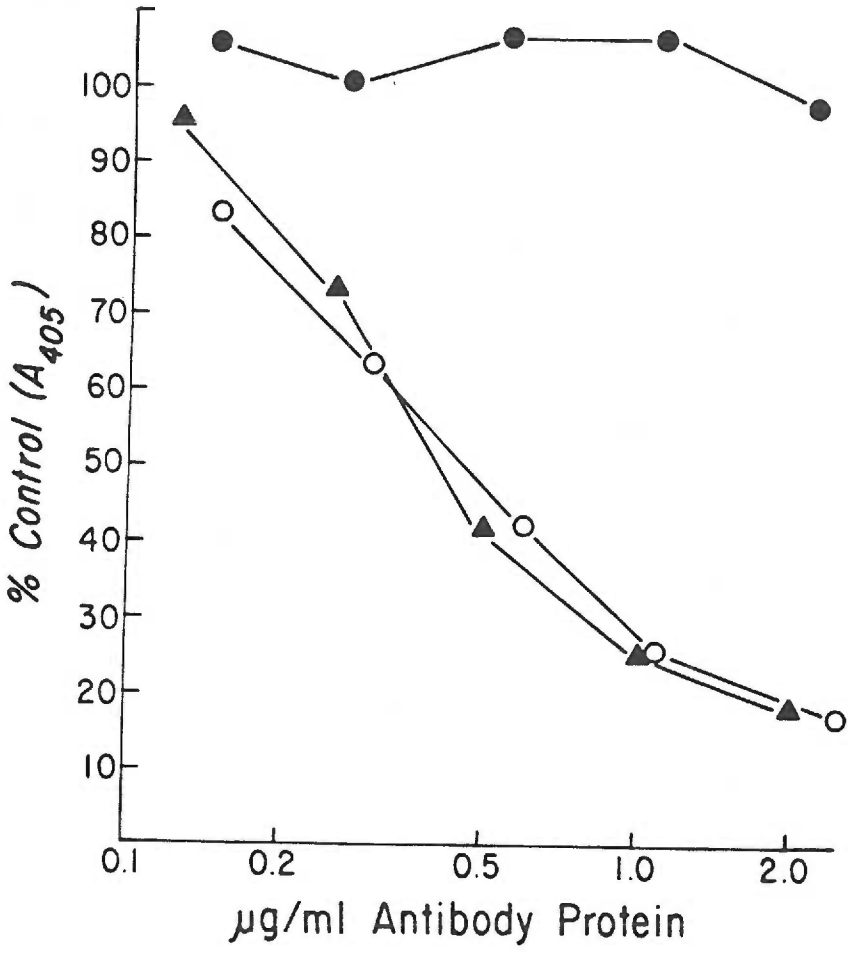
Serum Source	Type of Response[b]	NPPC/PC Ratio[a] IgM	IgA	IgG3	IgG1	IgG2a	IgG2b
Pool of 4	1°-d7	2.4	-	7.4	<.0006	-	-
	1°-d14	.08	2.9	7.6	<.0005	.04	4.7
	2°-d5	<.001	.58	.32	<.003	.003	<.0007
Pool of 3	1°-d11	0.25	2.7	6.0	0.6	4.1	20
Mouse μ1	1°-d10	4.4	3.1	4.5	2.8	-	4.4
	2°-d4	<.0008	.008	1.9	<.002	-	0.2
Mouse μ2	1°-d10	3.2	4.2	11.4	3.2	-	2.7
	2°-d4	1.7	6.4	4.8	.04	-	.0006
Long Term Memory[c]	2°-d5	3.6 ± 0.9	2.9 ± 1.0	2.4 ± 0.6	<.004	<.002	<.004

[a] Ratio of I_{50} values for NPPC and PC as in Table I.

[b] Mice were immunized with PC-KLH/CFA and bled at indicated times during the primary response (1°). The same mice were boosted 2 wks later with PC-KLH/CFA and bled at indicated times during the early secondary response (2°).

[c] For long term memory responses mice were primed and rested for at least 2 mo before challenge with PC-KLH/saline and were bled 5 d after challenge (data are taken from ref. 2).

Figure 1. Determination of T15 idiotype in Group I and Group II anti-PC antibodies. Various concentrations of T15 (Δ--Δ), Group I antibody (o--o), or Group II antibody (o--o), were mixed with enzyme conjugated T15 and tested for inhibition of binding of the T15 conjugate to anti-T15 coated plates as described in Materials and Methods.


100
90
80
70
60
50
40
30
20
10
% Control (A_{405})
0.1
0.2
0.5
1.0
2.0
µg/ml Antibody Protein

SUMMARY AND DISCUSSION

The primary antibody response of BALB/c mice to phosphorylcholine is dominated by B cell clones expressing a single pair of heavy and light chain variable regions regardless of whether thymus dependent antigens, such as PC-KLH, or thymus independent antigens, such as the pneumococcal vaccine R36A, are utilized (1). This variable region combination is serologically defined as the T15 idiotype (2) and is characteristic of immunoglobulin molecules having heavy chains belonging to the V_H-4 subgroup (3) and light chains of the V_K-22 subgroup (4). Heavy chain variable regions of PC-binding immunoglobulin molecules appear to be derived from a single germline V_H gene, the V1 gene, in combination with the J_H-1 gene and several different D segments (5). The corresponding light chain variable region gene(s) associated with the V_K-22 subgroup have not yet been studied although all known PC-binding myeloma proteins utilize the J_K-1 gene (6).

The objectives of this thesis were to determine the heterogeneity of the memory response induced by PC-KLH and to relate variable region expression of memory anti-PC antibodies to those of the primary response. The principal findings of this research were:

(1) Anti-phosphorylcholine memory involving IgM, IgA, IgG1, IgG2a, IgG2b and IgG3 antibodies can be developed by priming BALB/c mice with PC-KLH. This memory response was elicited *in vitro* and *in vivo* and was detected as plaque-forming cells and serum antibody.

(2) Variable region expression differed markedly among the different immunoglobulin isotypes produced in the memory response. Dominance

of the memory response by the T15 idiotype varied among the three immunoglobulin classes; while IgM and IgA plaque forming cells were mainly of the T15 idiotype IgG plaque forming cells were largely T15 idiotype negative. Fine specificity analysis revealed two major antibody populations: group I antibodies resembled TEPC 15 in specificity, exhibiting a similar or slightly greater avidity for PC than for NPPC, and constituted the majority of IgM, IgA and IgG3 antibodies; group II antibodies were distinct from TEPC 15 in fine specificity, possessed significant avidity only for NPPC and comprised most of the IgG1, IgG2a and IgG2b antibodies.

These observations raised the question concerning the origin of group II antibodies: were they derived from group I by somatic mutation or were they encoded by distinct variable region gene(s).

(3) Separation of group I and group II antibodies by affinity chromatography on PC-Sepharose confirmed the distinct fine specificity patterns of group I and group II antibodies and revealed a minor but significant group II IgM component. When the early secondary response to PC-KLH was examined high levels of group II IgM were detected. These demonstrations of group II IgM antibodies are inconsistent with the hypothesis that group II variable regions originate by somatic mutation activated by the class switch from C_μ to other constant region genes.

Although we have been able to rule out class switch-activated somatic mutation as the origin of group II variable regions, the origin of group II remains undetermined. One possibility is that one or more of the group II variable region germline genes (V_H, D, or J_H/V_L or J_L) differs from group I, conferring upon these antibodies their unique

specificity and the loss of the T15 idiotype. There is a precedent for expression of a novel V_H gene in the anti-PC response as discussed in the literature review, however this gene is typically expressed in BALB/c mice that are suppressed for the T15 idiotype (7). Our studies may provide the first instance in which different V_H genes are expressed in normal BALB/c mice during the course of an immune response to an antigen which elicits a T15 idiotype dominant anti-PC primary response.

Alternatively, group II antibodies may arise from group I by somatic mutation *prior to class switching*. Others have suggested that somatic mutation can occur in the absence of class switching (8). These alternatives are being expored currently utilizing a combination of B cell hybridoma, amino acid sequencing, and recombinant DNA technologies. We have generated a series of PC-KLH memory B cell hybridomas and one of these has fine specificity characteristics of group II and is negative for the T15 idiotype. Purified monoclonal group II antibodies and the DNA of these hybridoma cells will be studied to determine whether group II heavy and light chain variable regions are derived from the same or distinct germline genes as those utilized by TEPC 15 or any of other PC-binding myelomas.

Our observations on isotype expression in the PC-KLH response are consistent with those of Slack et al. (9). These investigators also detected a major IgG1 component along with IgG3 and IgG2 antibodies although they did not examine variable region expression by the IgG subclasses. The control of isotype expression during an immune response is a controversial topic. While the tandem organization of heavy chain constant region genes would predict that any set of V_H-D-J_H genes should

combine with any of the C_H genes several investigators, including us, have noted preferential class switching (10-14, paper 2). Screening of a variety of antigens including hapten conjugated to different carriers identified two factors which influence isotype expression: 1) a carrier effect was noted when TNP conjugates were examined; TNP-Ficoll (a TI-2 antigen) elicited primarily IgG3 antibodies with much smaller IgG1 and IgG2 components, TNP-LPS (a TI-1 antigen) elicited IgG2 and IgG3 exclusively, and TNP-KLH (a TD antigen) elicited a large IgG1 response with lower IgG2 and IgG3 responses (9), and 2) a hapten or determinant-specific effect was noted for carbohydrate antigens which induced a large IgG3 component regardless of whether the antigen used was thymus independent (R36A, dextran B1355) or thymus dependent (PC-KLH, group A streptococcus vaccine) (9). The influence of the activation signal on isotype switching has been examined in polyclonal antibody responses induced by lipopolysaccharide (A TI B cell polyclonal activator) or helper T cells specific for B cell surface antigens (a TD polyclonal activator) (12). The characteristic pattern of isotype expression induced by each of these polyclonal activators was attributed to the quality of the nonspecific stimuli delivered by thymus dependent as compared to thymus independent activators. The switch in isotype production has also been thought to be relatively T cell dependent (15,16). An immunoglobulin-specific helper T cell population appears to be required for IgG or IgE responses to thymus dependent antigens (17,18) and T cells as well as T cell factors have been noted to affect the pattern of isotype expression even to thymus independent antigens or mitogens (19,20). While these observations may on the surface appear

contradictory, it is possible that all of these components influence isotype expression, however, their activities may be directed to different B cell subpopulations. Slack and Davie have compared the isotype distribution to TI-1, TI-2 and TD antigens in T cell deficient nu/nu mice and their normal, heterozygous nu/+ littermates and found that isotype expression was unchanged for TI-1 (TNP-LPS) and TI-2 (TNP-Ficoll) antigens, while all IgG subclasses were reduced for a TD antigen (TNP-BSA) (21). Golding and Rittenberg have examined the effect of allogeneic T cell signals generated during a graft-vs.-host response on isotype expression in the primary response to TI-2 (TNP-Ficoll, TNP-dextran, TNP-levan), TI-1 (TNP-Brucella abortus), and TD (TNP-KLH) antigens (22). These nonspecific T cell signals greatly enhanced the expression of all isotypes in the TI-2 responses, with a shift away from the usual IgG3 dominance, while TI-1 responses were unaffected and TD responses were suppressed for all isotypes. Thus, T cells may be required for IgG isotype expression only for some antigens (17,18,21) and can influence isotype expression to other antigens (19,20,22). However, T cells do not appear to be the sole regulators of IgG isotype expression and it is possible that distinct B cell subpopulations responsive to different antigen forms become committed to the expression of specific isotypes without the involvement of T cell signals.

The development of the memory PC system will be useful for future studies on variable region-constant region associations by memory B cell subpopulations since at least two distinct variable regions with characteristic isotype preferences in the PC-KLH response have now been defined. Work is in progress to determine whether thymus independent

antigens such as R36A and PC-Brucella abortus elicit group I and group II memory antibodies which follow similar isotype associations as seen for the thymus dependent antigen PC-KLH. These studies may clarify whether these variable region-constant regions associations are totally the result of the phosphorylcholine (or diazophenyl phosphorylcholine) hapten or may be influenced by the nature of the carrier and its characteristic triggering properties.

We are also in the process of generating monoclonal anti-idiotypic antibodies against a group II hybridoma. This reagent will allow us to identify serologically a second variable region in the PC memory response and to determine whether antibodies falling within the group II fine specificity category may also be related idiotypically. If we are able to detect such a "public idiotype" shared among group II antibodies, we may be able to follow these clones, which are apparently severely underrepresented in the primary response, as they expand upon antigen priming to constitute a major component of the memory response to PC-KLH, and perhaps learn more about the regulatory interactions which permit this expansion to occur.

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