# Antigen Binding and Idiotype Analysis of Antibodies Obtained after Electroporation of Heavy and Light Chain Genes Encoding Phosphocholine-specific Antibodies: A Model for T15-Idiotype Dominance

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## Summary

Antibodies bearing the T15 idiotype dominate the murine primary immune response to phosphocholine (PC). Analysis of antigen binding of antibodies derived from V1:DFL16.1:$J_H1$ ($V_H1$) germline and N region-derived variant heavy (H) chains and κ22, κ24, and κ8 light (L) chains demonstrates that the T15H:κ22L (T15) antibody binds PC at least 20–40 times better than other antibodies derived from alternate germline forms of the $V_H1$ H chain and κ22, κ24, or κ8 L chains. To achieve affinities in the same range as the T15 antibody, κ24 and κ8 L chain–containing antibodies must have H chains derived from variant N region or somatically mutated $V_H1$ genes. Single amino acid differences at the VD junction of the various germline and N region variant $V_H1$ H chains dictate the L chain that can associate with the H chain to produce a PC-specific antibody. Several H:L combinations give rise to T15 or M167 idiotype-positive antibodies that lack specificity for PC, and single amino acid substitutions or insertions at the $V_H1$:D junction result in the loss of T15 or M167 idiotopes. Based on these observations, our data support a molecular model involving both preferential gene rearrangement and antigen-driven B cell selection to explain T15 idiotype dominance in the immune response to PC. In the absence of N region diversification, large numbers of neonatal B cells bearing the T15H:κ22L surface immunoglobulin M (sIgM) receptors would be selected and expanded by autologous or environmental PC antigen into the long-lived peripheral B cell pool.

The primary antibody response to PC in normal inbred mice is dominated by antibodies expressing H and L chain products of the $V_H1$ and $V_κ22$ germline genes (1, 2). These antibodies are identical to the antibody produced by the T15 plasmacytoma (3). The T15 clone of B cells is present in the mouse spleen at a higher frequency than any other individual clone of B cells and comprises >75% of all phosphocholine (PC)[1]-specific B cells in normal mice (4). The reasons for T15-Id dominance of the anti-PC immune response are not fully understood; however, idiotype network selection, binding-site affinity, and preferential V gene expression have all been suggested. A. J. Feeney (5) has recently shown that over 50% of the V1:DFL16.1 junctions produced in neonatal pre-B cells have prototypic T15 V-D junctional sequences, and has hypothesized that this preferential recombination of these germline genes would account for the dominance of T15 clones in the anti-PC repertoire. If the prototypic T15H chain is preferentially produced and this H chain can only form a PC-specific antibody with a κ22 L chain, the subsequent receptor-driven selection and amplification of this clone into a long-lived B cell pool could account for T15-Id dominance. This hypothesis is supported by our recent observation (6, 7) that PC-specific B cells in M167 H chain transgenic mice are selected and amplified by a receptor-mediated, antigen-driven process after they emerge from the bone

[1] *Abbreviation used in this paper:* PC, phosphocholine.

marrow. However, one would have to look for another explanation for T15-Id dominance if: (*a*) the T15 prototype H chain binds PC with a $K_a$ of $10^5$ or higher when associated with L chains other than κ22; or (*b*) antibodies containing alternate germline-derived $V_H1$ sequences can bind PC with an affinity in the same range as T15. From the data in this paper, it appears that these latter two conditions probably cannot occur in vivo.

X-ray crystallographic (8–10), amino acid sequencing (11, 12), and idiotypic (3) analysis of anti-PC binding antibodies (13, 14) have provided a detailed understanding of the correlations between antibody structure and antigen binding (for review see reference 15). Virtually all PC-binding mouse antibodies employ three L chains as represented by the BALB/c myeloma proteins T15 ($V_κ22$), M603 ($V_κ8$), and M167 ($V_κ24$) (8–20). These three L chains are only 45–74% homologous to one another in amino acid sequences; however, they all use $J_κ5$ and exhibit the Tyr-Pro-Leu amino acid sequence at position 94, 95, and 96 which provides the major L chain contact residues for PC (10, 15). The H chains of the prototype T15, M603, and M167 PC-binding antibodies, which provide the majority of PC contact residues, are derived from a single germline $V_H$ gene ($V_H1$) (21). This $V_H1$ gene segment is rearranged to DFL16.1 and $J_H1$ during somatic gene rearrangement, and the major differences between these prototype H chains exist at VD and DJ junctions (15, 17–20, 22, 23). Claflin (18, 22) and Feeney (19, 20, 23) and their collaborators have demonstrated that pivotal, "family-specific" amino acid changes occur at the V1:DFL16.1 junction of M603- and M167-like H chains which distinguish these antibodies from the germline T15 sequence. These VD-junctional residues appear to dictate the L chain which can associate with these variant H chains to form PC-specific antibodies. Thus, in virtually all M603-like antibodies, an invariant Asn is found at position 95H in place of the germline-encoded Asp, and in the majority of M167-like antibodies, an Ala has been inserted between Asp 95H and Tyr 96H of the T15 germline sequence (see Fig. 1). Although the GCA codon for Ala exists in the V1 germline gene, the insertion of an Ala or other amino acid at the VD junction of M167-like antibodies is generally accompanied by additional changes at VD or DJ junctions. This suggests that N region diversification is required to generate this PC-binding H chain sequence. The Asp to Asn change (a G:A transversion) which occurs in the M603-like antibodies also requires N region diversification of codon 95H in the V1 germline sequence (George, J., S. Penner, J. Weber, and J. L. Claflin, manuscript submitted for publication). Thus, the T15 prototype sequence may be the only H chain sequence derived from the joining of $V_H1$:DFL16.1:$J_H1$ gene segments in the absence of N region diversification or somatic mutation which binds PC with an affinity sufficient for clonal expansion by autologous or environmental PC antigens.

To address the molecular basis of T15-Id clonal dominance, we electroporated four different forms of the V1:DFL16.1:$J_H1$ genes in association with the $V_κ22$, $V_κ8$, or $V_κ24$ PC-binding L chain genes into the SP2\0 myeloma cell line. By analyzing the antibodies produced by these H-L chain pairs, we determined which germline and somatic variants of the $V_H1$ H chain gene product generated PC-binding antibodies when combined with these three L chains, and whether this PC binding was dictated by the family-specific, VD-associated, single-base changes discussed above. The results of these studies suggest that the T15H:κ22L antibody has the highest affinity for PC of the potential PC-binding $V_H1$ germline antibodies analyzed, and thus, even without preferential rearrangement of the T15H sequence, this H:L combination would be preferentially selected and expanded into the long-lived B cell pool.

## Materials and Methods

*Antibodies and ELISA.* The rat anti-$V_H1$ hybridoma T68.3, which recognizes all antibodies carrying a $V_H1$ H chain and the anti-T15-Id hybridoma T139.2 (24) were obtained from Dr. M. Scharff (Albert Einstein College of Medicine, Bronx, NY). The binding-site–specific rat anti–M167-Id hybridoma 28–5–15 (7), a nonbinding-site–specific anti-M167-Id (28–4–3), and the anti-$IgM^a$-allotype hybridoma (DS-1) (25) were obtained from Dr. D. Sieckmann (Naval Medical Research Institute, Bethesda, MD). The AB1.2 anti-T15-Id (26) and rat anti–mouse-κ (HB-58) hybridomas were obtained from the American Type Culture Collection (Rockville, MD). The polyvalent rabbit anti-T15-Id and goat anti–mouse IgM (μ specific) antibodies were produced as previously described (27, 28). ELISA for direct binding to PC-BSA–coated microtiter plates and anti-μ capture assays for detection of idiotypic and allotypic markers have been described (29). The antibody level in tissue culture supernatants was adjusted to 1 μg/ml based on the μ/κ capture assay. Anti-Id, anti-allotype, and direct PC-binding assays were then carried out on a five-place 1:5 dilution series of each supernatant. Standard curves for each assay were generated using affinity-purified T15-$Id^+$ or M167-$Id^+$ $IgM^a$-allotype antibodies from the HPCM2 or HPCM27 hybridomas (17), respectively. All assays were sensitive to a level of at least 10 ng/ml.

*H and L Chain Genes.* The M167-H and L chain ($V_κ24$) genes (30) were obtained from Dr. U. Storb (University of Chicago, Chicago, IL). Dr. P. Tucker (University of Texas, Dallas, TX) supplied the S107 (T15) H and L chain ($V_κ22$) genes (31). The M603-like (T15-Asn) and M167-like (T15-Ala) $V_H1$ genes were produced by PCR site-directed mutagenesis of a $V_HT15$:μ plasmid as described by Ho et al. (32). In brief, the $V_HT15$ gene was altered by changing the GAT at 95H to an AAT to produce the T15-Asn gene and by inserting a GCA codon after the GAT at 95H to produce the T15-Ala gene. The critical VDJ junctions of the H chain genes and the translated amino acid sequences of these genes are shown in Fig. 1. The $V_κ8$ L chain gene was cloned from the PC-specific hybridoma 206.8D3 (22) into λ EMBL4 arms as a 16.3-kb EcoRI genomic fragment. This gene contains 3 kb of sequence 5′ of the promoter and about 9.5 kb of sequence 3′ of $C_κ$ (Weber, J., and J. L. Claflin, manuscript in preparation).

*Electroporation of H and L Chain Genes.* Electroporation of linearized plasmids carrying H and L chain genes and the subsequent selection of geneticin (G418)-resistant cell lines has been described (7). Most electroporations were done using the SP2\0 myeloma (33), an H and L chain–deficient line, as the recipient cell line. However, cell lines 9 and 12 (see Table 1) were derived by electroporation of the designated H chain gene into a $V_κ8$ L chain-only hybridoma (301.2D7.7) (34), and cell line 11 was produced by

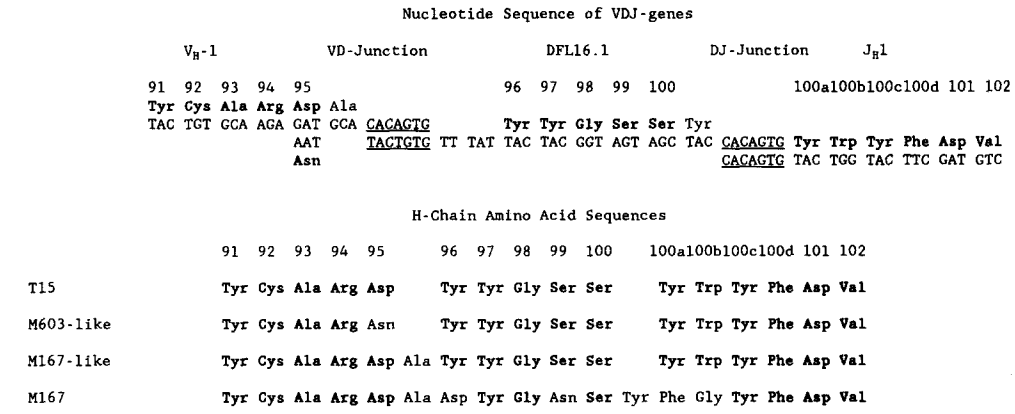


**Figure 1.** The nucleic acid sequence at the top represents the germline sequence for the 3′ end of $V_H1$ gene, the DFL16.1 diversity gene segment, and the $J_H1$ joining segment. The translated amino acid sequence is shown above the DNA sequence with the T15H chain sequence in bold. Alternate splicing, N region diversification, or somatic mutation of the T15 germline sequence gives rise to the M603-like, M167-like, or M167 protein sequences shown in the bottom part of this figure. The amino acid numbering was taken from Rudikoff (15).

electroporation of the $V_\kappa24$ L chain gene into a J558L:M167$\mu$ cell line obtained from Dr. Carol Sibley (University of Washington, Seattle, WA). This cell line produces an IgM$\lambda$, $V_H1$-Id$^+$ antibody that does not bind PC.

## Results

*Analysis of T15H Germline Antibodies.* The T15H chain gene (Fig. 1) was electroporated into separate aliquots of SP2\0

**Table 1.** *Characterization of Cell Lines Electroporated with PC-binding H and L Chain Genes**

| Cell line | Transfected V genes | $\kappa$ | IgM$^a$ | $V_H1$-Id | T15-Id | | | M167-Id | | PC-BSA |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | T139.2 | AB1.2 | PolyV | 28-5-15 | 28-4-3 | |
| 1 | VT15$\mu$ $V_\kappa22$ | + | + | + | + | + | + | − | − | + |
| 2 | VT15$\mu$ $V_\kappa24$ | + | + | + | − | − | − | + | + | − |
| 3 | VT15$\mu$ $V_\kappa8$ | + | + | + | − | − | − | − | − | ± |
| 4 | VT15$\mu$-Ala $V_\kappa22$ | + | + | + | + | − | + | − | − | − |
| 5 | VT15$\mu$-Ala $V_\kappa24$ | + | + | + | − | − | − | + | + | ± |
| 6 | VT15$\mu$-Ala $V_\kappa8$ | + | + | + | − | − | − | − | − | − |
| 7 | VT15$\mu$-Asn $V_\kappa22$ | + | + | + | + | + | + | − | − | − |
| 8 | VT15$\mu$-Asn $V_\kappa24$ | + | + | + | − | − | − | − | + | − |
| 9 | VT15$\mu$-Asn $V_\kappa8$ | + | + | + | − | − | − | − | − | + |
| 10‡ | V167$\mu$ $V_\kappa22$ | + | + | + | + | − | + | − | − | − |
| 11‡ | V167$\mu$ $V_\kappa24$ | + | + | + | − | − | − | + | + | + |
| 12 | V167$\mu$ $V_\kappa8$ | + | + | + | − | − | − | − | − | − |

* Cell lines designated with (+) in a given assay produced an antibody that bound within a twofold range of the antibody used to generate the standard curve; (±) a quantity 20–40-fold lower than the standard curve antibody was obtained at the 1-$\mu$g/ml dilution; and (−) nothing was detected at any dilution tested.

‡ The data for cell lines 10 and 11 have been previously published in reference 7.

cells along with one of the three dominant L chain genes from PC-binding antibodies (Table 1, cell lines *1–3*). As expected, the antibodies produced by all combinations of electroporated H and L chain genes expressed κ, $\mu^a$-allotype, and $V_H1$-Id markers. The homologous T15 cell line, which resulted from coelectroporation of the T15H and $V_\kappa22L$ genes, produced a PC-BSA binding antibody that was indistinguishable from the IgMκ HPCM2 (T15) hybridoma standard in both binding affinity and idiotype expression (Table 1, cell line *1*). When the T15H chain gene was combined with the $V_\kappa24L$ chain gene (Table 1, cell line *2*), the resulting antibody expressed both the binding-site–specific (28–5–15) and nonsite-specific (28–4–3) M167 idiotopes, but it failed to bind PC-BSA. Combining the prototype T15H chain gene with the $V_\kappa8L$ gene (Table 1, cell line *3*), resulted in an antibody that exhibits none of the H:L-dependent T15 or M167 idiotopes but that binds PC-BSA at a level 20–40-fold lower than the HPCM2 standard. The above results demonstrate that the prototype T15H chain can form an anti-PC antibody with at least one of the other PC-binding L chains, but the T15H:κ22L combination has a much higher affinity.

*Analysis of Germline T15-Ala H Chain Antibodies.* To address the question of whether or not other germline-derived $V_H1$:DFL16.3:$J_H1$ H chains besides the T15H can participate in the formation of PC-specific antibodies, the T15 gene was altered by PCR so that a GCA codon for alanine was inserted at the VD junction. The insertion of an extra amino acid at this position, generally Ala, occurs in almost all anti-PC antibodies belonging to the M167/M511 family (19, 20). This T15-Ala H chain gene was then electroporated into separate aliquots of SP2\0 cells along with one of the PC-binding L chain genes. The antibodies from the resulting G418-resistant cell lines (Table 1, cell lines *4–6*) were analyzed for binding to PC-BSA and idiotype expression. The antibodies composed of the variant T15-Ala H chain and either $V_\kappa22L$ (cell line *4*) or $V_\kappa8L$ (cell line 6) fail to bind PC-BSA. It is interesting that the T15-Ala-H:κ22L antibody expresses the T15 idiotopes detected by our polyvalent rabbit antibody and the rat T139.2 hybridoma, but the insertion of Ala between Asp 95H and Tyr 96H destroys the T15 idiotope detected by the AB1.2 anti-T15 antibody (Table 1, cell line *4*). The insertion of Ala at the VD junction of T15H not only destroys PC binding with the κ22 and κ8 L chains but creates an H chain that requires a κ24 L chain for PC binding. The T15-Ala-H:κ24L gene combination (Table 1, cell line *5*) results in an M167-$Id^+$ antibody that binds PC-BSA with an affinity 20–40 times lower than either the T15 (HPCM2) or M167 (HPCM27) standards. Additional amino acid changes at the DJ junction appear to be necessary to produce a $V_H1$:κ24 antibody with affinity for PC equivalent to T15. Thus, the M167 H chain, which has six amino acid changes in its CDR3 compared with T15, generates an antibody with much higher affinity than the T15-Ala H chain when combined with a κ24 L chain (Table 1, cell line *11*).

*Characterization of N Region-derived T15 Variant H Chain Antibodies.* Further evidence of the importance of family-associated VD-junctional H chain amino acids in determining L chain composition in PC-binding antibodies was obtained when the variant T15-Asn-H gene was electroporated into cells along with the three PC-binding L chain genes (Table 1, cell lines *7–9*). This H chain variant formed a PC-binding antibody with an affinity for PC-BSA equivalent to the HPCM2-T15 standard when combined with the κ8 L chain (cell line *9*). No detectable binding to PC-BSA was observed in either the T15-Asn-H:κ22L or T15-Asn-H:κ24L antibodies (cell lines 7 and *8*, respectively). It is interesting that all the T15-Ids analyzed, including the AB1.2 idiotope, remained intact in the non-PC-binding T15-Asn-H:κ22L antibody, whereas, the M167 binding-site–specific idiotope, 28–5–15, was lost in the T15-Asn-H:κ24L antibody.

The final three cell lines (Table 1, cell lines *10–12*), which were produced by combining the H chain gene from the M167 myeloma (Fig. 1) with the three PC-binding L chain genes, confirmed the observations made with the T15-Ala H chain (cell lines *4–6*). Thus, alteration of the T15 VD junction by insertion of an Ala after Asp 95H produced an H chain in which: (*a*) PC binding was achieved in association with a κ24 L chain (cell line *11*); (*b*) no binding to PC-BSA was seen when the M167 H chain was coexpressed in antibodies with either κ22 or κ8 L chains; and (*c*) the AB1.2 idiotope was lost in the antibody formed with the κ22 L chain (cell line *10*).

## Discussion

The above data show that: (*a*) the germline T15H chain forms the highest affinity anti-PC antibody when associated with the κ22 L chain, but it can form a low affinity anti-PC antibody when combined with the κ8 L chain; (*b*) N region generation of a T15-Asn 95H chain variant is required to obtain a high affinity PC-binding antibody with the κ8 L chain; (*c*) the germline T15-Ala H chain forms a PC-binding antibody of low affinity when combined with a κ24 L chain and additional N region or somatic mutations are required to obtain a high affinity anti-PC antibody with this H:L combination; and (*d*) the VD-junctional amino acids Asp 95H, Asn 95H, and Ala 96H are critical in determining which L chain generates a PC-binding antibody when associated with these variant H chains. Thus, κ22 requires the Asp 95H, κ24 requires the insertion of Ala or another residue at the VD junction, and κ8 functions optimally with the Asn 95H variant in formation of PC-binding antibodies. All of these observations lead to a molecular model to explain T15-idiotype dominance in the immune response to PC. T15-Id dominance occurs because: (*a*) during embryonic and early neonatal development, when the H chain repertoire is restricted to germline-derived junctional sequences (23), T15H appears to be the most frequently rearranged form of the $V_H1$:DFL16.1:$J_H1$ gene segments (5); and, (*b*) this T15 H chain forms the highest affinity germline anti-PC antibody in association with the κ22 L chain. Thus, even without the preferential recombination of T15-like VD junctions in neonatal pre-B cells, PC-driven expansion of these T15-$Id^+$ B cells into a long-lived B cell pool would make this the dominant PC-specific clone.

The lower affinity B cells expressing germline anti-PC receptors composed of T15H:κ8L or T15-Ala-H:κ24L molecules would not effectively compete with T15 clones for limiting PC antigen. The higher affinity M167- and M603-like anti-PC antibodies require N region diversification to generate their H chain sequences and would, therefore, be generated at a very low frequency in the newborn and adult bone marrow. The higher affinity phenylphosphocholine-specific B cells described by Stenzel-Poore et al. (35) do not compete with T15 because they are not selectively amplified by the endogenous form of PC recognized by $V_H1$ anti-PC antibodies. The ongoing development of H chain transgenic mice carrying somatic and germline H chain genes will allow us to test this model in bone marrow reconstitution studies where equal numbers of these clones can be allowed to compete for dominance of the peripheral anti-PC repertoire. However, the biological significance of the M167-like Ala-VD junctional change has been demonstrated in both M167 H chain (7) and κ24 L chain (20) transgenic mice. In the M167 H chain transgenic mice, all PC-specific B cells express the transgene-encoded H chain with an endogenous gene-encoded κ24 L chain, and in the L chain–transgenic mice, virtually all the PC antigen-induced antibodies express M167-like H chains with an extra VD-junctional amino acid, usually Ala, plus accompanying N region changes. Thus, in these M167 H or L transgenic mice, anti-PC antibodies with the appropriate endogenous L chain or variant H chain are being selected by PC antigen. We are currently producing T15-Ala H chain–transgenic mice to determine the lower affinity limits for antigen-driven selective expansion of PC-specific B cells in normal mice, and for clonal deletion of PC-specific B cells in *xid* mice.

The lack of PC binding by the T15H:κ24L antibody concurs with the H:L chain reconstitution studies of Sher et al. (36). When the S107 (T15) H chain was combined with the Vκ24 L chain from the M511 myeloma, they found that the recombinant antibody bound PC <10 and 30% as well as the parental S107 and M511 antibodies, respectively. This residual binding could have been due to S107 L chain contamination. Furthermore, T15H:κ24L-bearing B cells are not selected by antigen in the peripheral lymphoid tissues of IgAκ transgenic mice (37) expressing these genes (Kenny J. J., C. O'Connell, G. Guelde, J. Hurst, D. Lo, and D. L. Longo, manuscript in preparation). These mice make predominantly T15-Id$^+$ anti-PC antibodies in which the IgA transgene product is coexpressed with endogenous κ22 L chains. Since we have previously shown that peripheral selection of B cells appears to be an antigen-driven process (7), these data in IgAκ transgenic mice argue strongly that the T15H:κ24L antibody is also not PC binding in vivo.

If N region diversification is absent in the fetal and neonatal mouse as suggested by Feeney (23), then our data suggest that there are only three germline H:L combinations that produce anti-PC antibodies, T15H:κ22L, T15H:κ8L, and T15-Ala-H:κ24L. Of these, T15H:κ22L has by far the highest affinity for PC and should be selectively expanded by autologous or environmental antigen. When the T15H:κ22L clone was suppressed during neonatal development by injection of anti-T15-Id antibodies, the low affinity germline clone T15H:κ8L (HPCM3) (17), which binds PC with a Ka 10 times lower than the T15 prototype antibody, was activated after immunization with PC. The decreased affinity of the T15H:κ8L antibody compared with the T15H:κ22L antibody could be due to the extra charge introduced by Asp 91L in the former.

Padlan et al. (10) have suggested that the negatively charged carboxylate side chains of Asp 91L in M603 or Asp 95H in T15 and M167 are important for PC binding because of their interaction with the positively charged trimethylammonium moiety of PC. A higher affinity antibody is generated when the Asp 95H in the T15H gene is mutated to Asn as shown in the M603-like T15-AsnH:κ8L antibody (Table 1, cell line *9*). This latter mutation restores the charge balance in the choline binding pocket. It is interesting that no additional N region or somatic changes were necessary to achieve good binding to PC-BSA even though most M603-like antibodies have additional N region changes (18, 22). No detectable binding to PC-BSA was observed in either the T15-Asn-H:κ22L or T15-Asn-H:κ24L antibodies (cell lines 7 and *8*, respectively). This observation confirms the key role that Asp 95H plays in forming a salt bridge with the choline moiety of PC (*9*) in both the high and low affinity binding of the T15H:κ22L and T15:κ8L cell lines discussed above. The loss of this ionic bridge in the T15-AsnH:κ22L and κ24L antibodies is offset in the T15-Asn-H:κ8L antibody by the negatively charged Asp 91L in the κ8 L chain which cannot only reestablish the salt bridge with the choline moiety of PC, but also forms a stabilizing hydrogen bond with the Asn at 95H (9, 10).

The data in this paper support the hypothesis that the B cells bearing the T15-Id dominate the immune response to PC because this H:L combination has the highest affinity for PC of the potential germline anti-PC antibodies, and these T15-Id$^+$ B cells are thus selectively amplified into the long-lived peripheral B cell pool by autologous or environmental antigen during early neonatal life. These long-lived neonatally-derived T15H:κ22L B cells dominate the immune response to PC throughout adult life. As shown by Feeney (5), the adult bone marrow does not appear to regenerate the germline T15H sequence because of extensive N region diversification, and our data show that N region-modified T15H chains produce T15-Id$^+$ antibodies that do not bind PC. Thus, the production of T15-Id$^+$ B cells capable of binding PC appears to be largely restricted to the perinatal period.

The above observations and our previous publications (6, 7) demonstrating antigen-driven positive and negative selection of PC-specific B cells in M167 H chain–transgenic mice strongly suggest that antigen, rather than an anti-Id network, drives the formation of the peripheral B cell repertoire.

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