Repertoire Shift in the Humoral Response to Phosphocholine-Keyhole Limpet Hemocyanin: $V_H$ Somatic Mutation in Germinal Center B Cells Impairs T15 Ig Function

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Phosphocholine (PC)\(^3\) is an epitope present on *Streptococcus pneumoniae* and a number of other pathogenic microorganisms (reviewed in Ref. 1). In the primary Ab response to PC conjugated to keyhole limpet hemocyanin (PC-KLH), BALB/c mice produce large amounts of T15 idiotype Ab, which is highly protective against pneumococcal infection (2, 3). These Abs bind to free PC and are classified as group I Abs (4, 5). During the late primary and early secondary responses to PC-KLH, a shift in the Ab repertoire from group I to group II occurs. Group II Abs possess a different fine specificity and use a diverse set of V gene combinations that differ from group I (reviewed in Ref. 6). Group II Abs bind \(^p\)-nitrophenyl PC (NPPC) (4) or aminophenyl PC (7) but bind weakly or not at all to phosphocholine itself, (4, 8) and are not protective against pneumococcal challenge (9–11). Understanding the fate and location of T15\(^+\) B cells provides an important model for dissecting the molecular basis of repertoire shift and the maintenance of protective immunity.

T15 Id\(^+\) Abs are derived from CD5\(^+\) lineage B cells (7, 12–16) with precursors distinct from group II Abs (17–19). Reconstitution experiments have suggested that CD5\(^+\) cells cannot form germinal centers (20), which might preclude T15 from accumulating mutations. The ability to participate in germinal center reactions remains unclear since Miller et al. (21) have reported T15 Id\(^+\) germinal centers from which they could amplify the canonical V\(_h\)-DFL16.1-JH1 rearrangement of T15; however, the presence of the T15 V\(_k\)-22-Jk5 rearrangement was not formally demonstrated.

Because of its importance in protective immunity, and lack of evidence for somatic mutations that enhance Ag binding, it has been suggested that the T15 Ab may be unable to tolerate substantial somatic mutation in the H chain (22, 23) or L chain (24). This would limit affinity maturation and lead to loss of T15 dominance due to the appearance of other Abs more tolerant of somatic mutations. Previously, we introduced random mutations into the T15 H chain CDR2 region in vitro and expressed the mutant H chains with the germline T15 L chain. Of 46 T15 mutants, none displayed significantly increased binding for PC-containing compounds while ~50% of the multisite mutants had significantly impaired Ag binding and 10% of the mutants were not secreted from the transfected cells (25–27). These results support the notion that T15 may not be able to tolerate mutations. Our in vitro studies did not indicate whether similar deleterious somatic mutations would occur normally in vivo or whether they might contribute to repertoire shift. These issues are examined herein.

We confirm that T15 Id\(^+\) germinal centers can be found during a primary response to PC-KLH and that these germinal centers contain rearranged V\(_h\)-DFL16.1-JH1 as reported by Miller et al. (21). We extend their findings to demonstrate rearranged V\(_k\)-22-Jk5 genes in the same pool of cells from single microdissected T15\(^+\) germinal centers. The repertoire shift occurred by the late primary response as group II Abs increased to predominate in the serum. To assess the effects of somatic mutation on V\(_h\)1 genes
obtained from primary response germinal centers, six variant H chains were coexpressed with the unmutated T15 L chain. Four of the tested mutants containing one to four amino acid substitutions either lost binding to PC-protein or failed to pair with the T15 L chain. These results highlight the harmful effects of mutation on Ig function and are the first evidence that mutation occurring in germinal centers can affect Ig assembly and secretion. We discuss the molecular mechanisms of how somatic mutations after binding and secretion of Ig in the context of a T15 structural model and how these findings contribute to understanding of repertoire shift.

Materials and Methods

Animals and immunization

PC-KLH was prepared as described (28) and had a hapten to protein molar ratio of 40 per 100,000 m.w. KLH. Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. For primary responses, 8-wk-old mice were immunized once i.p. with 60 μg of PC-KLH precipitated in alum. For secondary responses, mice were immunized i.p. with 100 μg of PC-KLH in saline. Serum and spleens were obtained from euthanized mice on days 3, 5, 7, and 13 after primary immunization or on days 47 and 55 (7 and 13 days after secondary immunization). Spleen samples were processed for immunohistochemistry as described (29).

T15 and anti-PC ELISA

Anti-PC serum Ab was quantified by ELISA using dilutions of purified T15 Id– hybridoma Ab PCM11 (6) as a standard. Dilutions of serum in PBS containing 1% BSA and 0.04% sodium azide were incubated in ELISA wells coated with PC/BSA (1 μg ml−1). Inhibition by PC or NPPC was determined by preincubating a 1/10000 dilution of serum with 0.01 M free hapten. After washing, wells were incubated with a mixture of purified goat anti-mouse κ and goat anti-mouse λ coupled to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The concentration of anti-PC Ab was determined by comparison of the A410 values to the PCM11 standard curve.

The amount of T15 Id– IgM Ab present in serum samples was quantified by ELISA using PCM11 hybridoma Ab as a standard. Dilutions of serum were incubated in wells coated with purified anti-T15 Ab A1B-2 (30) at 1 μg ml−1, followed by incubation with purified goat anti-mouse IgM coupled to alkaline phosphatase (Zymed Laboratories, South San Francisco, CA). The amount of T15 Id– IgG in serum was determined in an ELISA by comparison to a pool of Abs containing T15 IgG2b (25) and T15 Id– hybridoma Abs PCG1-7 (IgG1), PCG2a-2 (IgG2a), and PCG3-1 (IgG3) derived from spleen cells of BALB/c mice immunized with PC-KLH (6). Serum dilutions were incubated in wells coated with purified TC54 (31) at 0.5 μg ml−1, followed by incubation with rat anti-mouse IgG coupled to alkaline phosphatase (Zymed Laboratories).

Immunohistochemistry

Goat anti-T15 serum was obtained from Dr. J. Kenny (National Institutes of Health, Bethesda, MD). The Ab was affinity purified on a T15-Sepharose 4B column and adsorbed on mouse IgMx and mouse IgGlx coupled to Sepharose 4B. The goat anti-T15 Id strongly recognized T15 Ab but also had minor reactivity with the group I myeloma Abs M511 (16-fold lower), M603 (196-fold lower), and M167 (66-fold lower). No reactivity was observed with the group I myeloma Abs M511 (16-fold lower), M603 (196-fold lower), and M167 (66-fold lower). No reactivity was observed with the group II hybridomas D16 (1y2.e, 26), M365 (6y2, λ) (32), PCG1-1 (γ1, κ), (6), or with IgAa of unrelated specificity (Zymed Laboratories). Confirmation of T15 Id staining in germinal centers was performed using biotinylated anti-T15 Ab A1B-2 which recognized the T15 H and L chains for binding (30). Germinal centers were identified by staining with peanut agglutinin (PNA)-HRP (EY Laboratories, San Mateo, CA). For staining, spleen sections were rehydrated in PBS, pH 7.4, and blocked with PBS containing 1% BSA. HRP reactivity was visualized using 0.4 mg ml−1 3-amino-9-ethylcarbazole, and alkaline-phosphatase-labeled Ab were detected with naphthol-AS-MX phosphate and Fast Blue BB salt (Sigma-Aldrich, St. Louis, MO) as described (29).

Microdissection of cells, DNA amplification, and sequencing

Cells from T15 Id–, PNA– germinal centers were microdissected from spleen sections using a micromanipulator (Narishige MHW-103, Tokyo, Japan) controlled micropipette as described (29). Microdissected tissue, containing groups of 10–50 cells, was digested in proteinase K containing buffer (33) and the DNA was subjected to two rounds of PCR in a 9700 GeneAmp PCR System (Applied Biosystems, Foster City, CA). Each round of PCR consisted of 38 cycles (94°C for 1 min, 58°C for 40 s, 72°C for 1 min) with the last incubation at 72°C for 7 min. PCRs were conducted in a 50-μl volume containing 200 μM dNTP, 0.01% gelatin (Sigma-Aldrich), and 2.5 U PuF Turbo DNA polymerase, and 1× cloned Pu DNA polymerase reaction buffer (Stratagene, La Jolla, CA). The first round of amplification contained primers (10 pM of each) to amplify Vj1-HJI and Vε22-ε5 rearrangements. Primers were also included for the RAG-1 gene to determine the PCR error frequencies. All primers included in the first round were: Vj1(for) no. 3, 5′-GGT TGT GCA TAT TGT TGT AAC AGG G-3′; Vj1(rev) no. 3, 5′-GGG AGA TCT GAG AAT ATC TTT TCC CG-3′; Vε22-ε5 no. 1, 5′-AAA CTG AGC AGT GTC AAA CCA CAG CAG G-3′; Jε5nt no. 1, 5′-CGT CAA CTG ATA ATG ACC CCT CTC C-3′; RAG-1for no. 1, 5′-ATG AAC TCA CTG GAC ACC ACG G-3′; RAG-1rev no. 1, 5′-GCC GAA ACC GCC TAA ACA AGC-3′. Vj1, Vε22, and RAG-1 were amplified in separate tubes in the second round using internal primers (20 pM each): Vj1(for) no. 4, 5′-TAC GGT TCA TGG TGA TG-3′ and RAG-1rev no. 4, 5′-CCA GCT TAC CTG AGG AGA CG-3′, or Vε22 no. 2, 5′-AGC AAA TTT TAA CTG CTG CTA TAT TCC G-3′ and Jε5nt no. 2, 5′-CGG AAA CAT CAT GAC GAA AAC TGT G-3′, or RAG-1for no. 2, 5′-TCA TCG AGA CAG TCT CCT CC-3′ and RAG-1rev no. 2, 5′-TAC GGT TCA TGG TGA TG-3′. DNA from PCRs was purified from agarose using the QiAquick Gel Extraction Kit (Quiagen, Valencia, CA). PCR products were cloned into the pSBlue-1 vector and transformed into Nova Blue competent cells using the Perfectly Blunt Cloning Kit (Novagen, Madison, WI). DNA was purified and subjected to automated, fluorescent DNA sequencing (ABI 377; Applied Biosystems) in both directions. As a positive control, 10 T15 Id– hybridoma cells were plated in lysis buffer and VDJ and VIJ rearrangements amplified, cloned, and sequenced as described above. As a negative control, microdissection pipette tips were dipped in buffer surrounding tissue sections and subjected to PCR amplification in each experiment. No VDJ, VIJ, or RAG1 PCR products were observed.

Nucleotide sequence accession numbers

Sequences of the cloned Vj1 and Vε1 genes have been deposited in the GenBank database under accession numbers AY194179-AY194222.

Expression of mutant Vj1/ε1 genes

Wild-type (WT) and mutant VDJ rearrangements were amplified by PCR with primers Xho/VH1, 5′-ATC TTA TCT CGA GCA GOT ATC AGC TGT GAG GTC AAG C-3′ and JH1/S107 5′-ATC TTA TCT CGA GCA GOT ATC AGC TGT GAG GTC AAG C-3′ to introduce the XhoI and NcoI restriction sites. PCR products were digested and ligated into the pSV2-S107 expression vector containing the BALB/c γ2b C region gene and the unmutated T15 L chain gene (34). The vector was engineered to contain XhoI and NcoI restriction sites flanking the T15 VDJ region, called pSV2-S107(XN). Transfectants of the WT and mutant Abs were obtained by transfecting SP2/0 cells as described (25). Stable transfectants secreting Ab or producing intracellular H and L chains as determined by ELISA were saved for further analysis.

Ag-binding assays

Secreted Ab was purified from cell culture supernatants using protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden), then dia lyzed against PBS-containing 0.04% sodium azide. Binding of T15 mutant Abs relative to WT was determined by ELISA as described (35). Abs were tested for binding to PC-histone, 6-O-(phosphocholine) hydroxyhexanoate (EPC)-BSA, S. pneumoniae strain R36A, Trichinella spiralis, and Ascaris suum as described (35).

Secretion assays

Stable transfectants were plated in triplicate at 1 × 10⁶ cells ml−1 of tissue culture medium in a 12-well plate. After a 4-h incubation, culture supernatants and cell lysates were determined by sandwich ELISA as described (26). Briefly, to determine H + L in the supernatant or lysate, ELISA plates were coated with rabbit anti-mouse IgG2b (Zymed Laboratories) and the amount of bound Ig from the lysate or supernatant was determined by a secondary alkaline phosphatase-conjugated, goat anti-mouse κ Ab (Southern Biotechnology Associates). All antisera were used at a 1/10000 dilution. Standard curves were generated using affinity purified Ab from transfected SP2/0 cells producing WT T15 Ab.
Results

Repertoire shift in serum Abs occurs in the late primary Ig response of BALB/c mice immunized with PC-KLH

Mice were immunized with PC-KLH and serum was collected on the days indicated (Fig. 1A). The concentration and specificity of serum Ab is shown. The anti-PC Ab level averaged 175 ± 87 μg ml⁻¹ by day 7, a 44-fold increase over preimmune levels, while T15 Id⁺ Ab averaged 122 ± 29 μg ml⁻¹, a 23-fold increase above preimmune levels. Consistent with previous findings, the majority of PC-BSA binding, as determined by hapten inhibition ELISA, was inhibited by 10⁻² M free PC (92% at day 7 and 98% at day 13) indicating that most Ab produced early in the primary response has a group I binding phenotype (Fig. 1B). However, by day 42 of the immune response only 17% of anti-PC Abs could be inhibited by free PC. Those anti-PC Abs that were not inhibited by free PC were inhibited by NPPC and thus are characteristic of group II Abs by free PC. Those anti-PC Abs that were not inhibited by free PC had a group I binding phenotype (Fig. 1B). However, by day 42 of the immune response only 17% of anti-PC Abs could be inhibited by free PC. Those anti-PC Abs that were not inhibited by free PC were inhibited by NPPC and thus are characteristic of group II Abs that develop during repertoire shift in BALB/c mice after PC-KLH immunization (Fig. 1C).

Day 7 germinal centers stain with anti-T15 Id⁺ Abs and contain rearranged \( \text{V}_{\mu}1-\text{DFL}16.1-\text{JH}1 \) genes and \( \text{V}_{\kappa}22-\text{Jk}5 \) genes

To follow the location of T15 Id⁺ B cells in vivo, spleen sections were stained with a polyclonal anti-T15 serum or with biotinylated monoclonal AB1-2. The same or adjacent sections were also stained with PNA which is a marker of germinal center B cells. Germinal centers were not observed on days 3 and 5, but by day 7 after immunization, PNA⁺ germinal centers were numerous and 77% were T15 Id⁺. A representative germinal center is shown (Fig. 2A). The anti-T15 staining was specific, as it was completely inhibited by preincubation of spleen sections with the T15 Id⁺ mAb, PCM11 (Fig. 2B). In addition to the Id⁺ germinal centers, numerous foci of T15 Id⁺ cells were also present outside of germinal centers (data not shown).

To determine whether the T15 Id⁺ cells contained canonical T15 VDJ and VJ rearrangements, groups of cells from Id⁺ germinal centers were microdissected from spleen sections and rearranged H and L chain genes were amplified using nested primers. T15 VDJ and VJ rearrangements were readily amplified from germinal centers of the primary response. DNA from 19 microdissected germinal centers resulted in 19 positive T15 VDJ amplifications and 16 VJ amplifications.

Low levels of somatic mutations are present in \( \text{V}_{\mu}1 \) and \( \text{V}_{\kappa}22 \)

To determine whether somatic mutations were introduced during the primary response to PC-KLH, 131 \( \text{V}_{\mu}1 \), and 57 \( \text{V}_{\kappa}22 \) rearrangements were cloned and sequenced. Day 7 was chosen as this time point would capture B cells undergoing early events in mutation and selection within splenic germinal centers (36). Consistent with this, many sequences were found to be unmutated; however, 34% of \( \text{V}_{\mu}1 \) clones and 37% of \( \text{V}_{\kappa}22 \) clones contained one to four mutations in the V region. Possible “N” additions at the V/D junctions were not scored as V region mutations but are shown in Fig. 3. The overall mutation frequency, calculated for amino acids 1–95, for \( \text{V}_{\mu}1 \) clones was 1/484 bp and for \( \text{V}_{\kappa}22 \) clones was 1/785 bp. The PCR error frequency was 1/10608 bp, estimated by sequencing of the RAG-1 gene amplified in the same PCRs indicating that nearly all (~95%) of the mutations are introduced by somatic hypermutation. Fig. 3 shows the unique T15 H chain V regions obtained from day 7 germinal centers. Most VDJ rearrangements use the T15 canonical \( \text{V}_{\mu}1-\text{DFL}16.1-\text{JH}1 \) gene segments typical of T15, but 4 of 34 unique sequences show alternative D region amino acids. Four groups of clones from mouse 9 appear to be related, as they were amplified from the same germinal center cell populations, share complementarity determining region (CDR)3 junctions, and have shared mutations (G1.1 and G1.2; G1.8 and G1.9; G1.37 and G1.38; G4.19 and G4.23). One clone (G4.2, mouse 8) has a deletion in the internal heptamer region (37) resulting in stop codons. Four other unique sequences from mouse 9 also included stop codons: one in CDR2 (G9.1), one in FW3 (G1.4), and two in JH1 (G1.1 and G1.2). The replacement to silent ratios (R:S) for the \( \text{V}_{\mu}1 \) CDRs was 14:0 and was 3.8:1 for the FW regions.

The mutated T15 L chain V regions are shown in Fig. 4. Although these clones are also mutated by day 7, fewer unique L chain clones were found (17.5%) compared with the sequenced H chain clones (26%). One stop codon was generated in the CDR1 region (G2.48, mouse 9) in a clone that is probably related to clone...
G2.43. The R:S ratio in the L chain was 4:1 in the CDRs and 4:1 in the FW regions.

To determine whether T15 B cells with mutated sequences were also present in germinal centers after the secondary response, PCR analysis was conducted on germinal centers on days 7 and 13 after boosting with PC-KLH. At these time points, germinal centers were numerous and large in size (data not shown). Only two VH 1 amplifications and no V\(\mu\)/H926022 amplifications were obtained from 26 individual germinal centers in PC-KLH-boosted mice, even though RAG-1 was amplified in 23 of 23 PCRs. Of the two VH 1 amplifications, all sequences were germline (n/H1100520). PCR conditions were appropriate for amplification as VH 1 and V\(\mu\)/H926022 rearrangements were amplified from 9 of 10 Id\(^+\) foci. These data indicate that B cells with V1-JH1 and V\(\mu\)/H926022-JH5 rearranged V genes were infrequent in secondary response germinal centers but could be readily identified outside of germinal centers.

Deleterious somatic mutations in T15 V\(\mu\) affect Ag binding

To determine whether deleterious mutations other than stop codons were being generated in the primary response germinal centers, we identified a set of 7 VH 1 clones including WT to put into the pSV2-S107 (XhoI/NcoI) expression vector, replacing the S107 WT VH 1 with the cloned VH 1 rearrangements and retaining the unmutated S107 WT V\(\mu\)/H926022 (Fig. 5). The S107 WT V\(\mu\)22 gene was used, as to our knowledge, the canonical T15 H chain rearrangements that we chose for expression studies have not been

FIGURE 4. Sequences of amplified V\(\mu\)22 to Jk5 rearrangements. The sequences are compared with germline V\(\mu\)22 and to the T15 myeloma L chain. The number of identical clones analyzed are listed in parentheses after each sequence.
reported to pair with other L chains. The specific VH1 clones were chosen because they might have deleterious mutations as determined by analysis of the mutation in the context of the T15 structural model and the amino acid substitution frequency within the Kabat database of Ig sequences (38, 39). After transfection into SP2/0 cells, transfectants were assayed for ability to be secreted and for ability to bind Ag. WT and four mutant transfectants secreted Ab and were assayed by ELISA for binding to a variety of PC-containing Ags (Fig. 6). PC is coupled to the histone protein via a nitrophenyl linker, as is PC in the PC-KLH used for immunization. EPC-BSA has a linear nine-carbon linker joining the PC hapten to the protein carrier (9). The three natural PC-containing pathogens, S. pneumoniae strain R36A, T. spiralis, and A. suum, were chosen because they differ from each other in the form of PC Ag displayed on the surface of the organism (40). Compared with WT, two of the Abs, VH1-1 and VH1-10, have lost the ability to bind to any of the PC-Ags present in the panel. Two other Abs, VH1-2 and VH1-4, retain binding ability but show differential recognition of the panel of PC Ags, exhibiting very poor binding to S. pneumoniae R36A, T. spiralis, and no detectable binding to A. suum. To verify that the VH1-2 and VH1-4 Abs can recognize the PC hapten, we inhibited the binding to PC-histone using different concentrations of free PC hapten. The IC50 (hapten concentration needed to inhibit Ab binding by 50%) values were 0.005 mM for WT, 0.02 mM for VH1-2, and 0.003 mM for VH1-4. The similar IC50 value of VH1-4 to WT indicates that VH1-4 can recognize PC as well as WT. However, the single CDR2 mutation T56A in VH1-4 modifies the Ab so that it no longer has a broad Ag recognition range, but has become more specific for PC in the form of the immunizing Ag. A similar restricted pattern of recognition of PC-containing Ags is seen with VH1-2 which has changes in CDR3 due to use of different D region amino acids.

Deleterious mutations in T15 V\textsubscript{H}4 affect Ab secretion

Two transfectants (VH1-6 and VH1-8) consistently failed to secrete Ig upon primary screening. At least 50 antibiotic resistant clones of each mutant were screened from three separate transfections. Stably transfected secretion of WT amounts of Ig (Table I). The secretion defect was not due to a defect in H chain or L chain synthesis as intracellular levels were similar to or above WT as determined by ELISA. To confirm these results, metabolic labeling of stable transfected cells was conducted for 4 h with [35S]Met/Cys. Abundant intracellular H and L chains were found for mutants VH1-6 and VH1-8 (data not shown). The presence of the mutations in the transfected cell mRNA was confirmed by RT-PCR and direct sequencing of the PCR products (data not shown). These results indicate that VH1 genes, harvested from germinal centers, can contain mutations that impair assembly and secretion with the Vk22 L chain.

Discussion

The molecular basis for loss of dominance of T15 Id* Abs during the late primary and early memory response to PC protein is not completely understood. We demonstrate in this study that T15 Id* cells are present within PNA+ germinal centers during the primary immune response to PC-KLH. Both rearranged VH1-DFL16.1-JH1 genes as well as rearranged Vκ22-Jκ5 genes were readily amplified from microdissected single germinal centers before the reperitoneal shift in serum Ab. Although we have not formally demonstrated the presence of T15 VH and Vκ genes within a single cell, the Vκ22-Jκ5 L chains form functional PC binding sites only when paired with the T15 H chain (41). Taken together, the amplification of canonical T15 H and L rearrangements within cells of the same germinal centers supports our immunohistochemical identification of T15 Id* cells. These findings are consistent with other studies in which T15 Id* cells were observed in germinal centers 7 days after immunization with other types of PC-containing Ags, including EPC-KLH (21) and S. pneumoniae R36A (42). Our results

| Transfected Construct | Supernatant H+L% | Intracellular H (%) | Intracellular L (%) |
|-----------------------|-----------------|--------------------|--------------------|
| T15 WT                | 100             | 100                | 100                |
| VH1-6 (H11)           | <1              | 482                | 349                |
| VH1-8 (D4)            | <1              | 635                | 73                 |

* T15 WT or mutant H chains constructs were transfected into SP2/0 cells. Triplicate cultures of stable transfected were cultured for 4 h in fresh medium and the concentrations of secreted and intracellular Ig were determined as described in Materials and Methods. Data are representative of at least three separate experiments.
extend these findings as the presence of Vx22-Jx5 rearrangements had not been formally demonstrated. The T15 Id⁺ PNA⁺ cells appear to be active participants in the germinal center reaction as somatic mutations were observed in the amplified VDJ genes and VJ genes at a frequency comparable to mutation seen in other T cell-dependent germinal center responses (36, 43) and is higher than that seen in T cell-independent germinal center responses (44). Collectively, our results argue that repertoire shift occurs even though mutated T15 Id⁺ cells are present in the microenvironment of the germinal center early in the immune response.

Why do T15 Id⁺ B cells enter germinal centers during the primary response yet fail to maintain idioype dominance?

Ab affinity dependence competition among B cell clones in transgenic and Ig-targeted mice is an important factor limiting the persistence of low affinity cells within germinal centers (45, 46). In the absence of competition, both high and low affinity B cells are equally able to respond to Ag and form germinal centers; however, in adoptive transfer competition experiments only high affinity B cells selectively accumulate during the primary response. Our finding of T15⁺ cells undergoing mutation within germinal centers early in the primary response suggests that competition between group I and group II precursors may be occurring within germinal centers. Group II precursors may be at an initial disadvantage as their precursor frequency is much lower than group I precursors (17–19). In addition, the affinity of germline and some mutated group II appear to be the same or lower than 1.8 × 10⁵ M⁻¹, the affinity of unmutated T15 for NPPC (8). This argues against initial affinity providing a competitive advantage for group II Ab precursors. However, the contribution of the carrier molecule to binding could be significant. For example, the affinity of the M3C65 group II Ab for NPPC conjugated to peptide is ~110 times greater than the affinity for NPPC (32). Thus, the apparent similarity of affinity between group I and group II precursors for NPPC may not fully reflect the affinity of group II B cells for the carrier determinants present on the selecting ligand. In addition, the selective forces in vivo are likely to be complex as the B cell receptor is sensitive to binding kinetics (k_{off} and k_{on}), avidity, and epitope density and organization (47–52). Clearly, group II Abs develop higher affinity binding for the PC carrier as recurrent mutations can increase the affinity for the PC-protein complex up to 1000-fold greater than T15 (32, 53). In the M3C65-NPPC cocystal, somatic mutations in the L chain directly enhance contact with the nitrophenyl group linking PC to KLH (32). In addition, group II Abs exhibit a higher efficiency for clonal expansion (19) suggesting that the potential of mutated group II Ab precursors to be favorably selected and expanded may provide a critical selective advantage.

Deleterious somatic mutation within germinal centers

It has been previously proposed that T15 Abs are unable to form better combining sites in response to somatic mutation (22–25, 54). T15 Abs are reported to undergo little affinity maturation (23, 55) and in vitro studies have demonstrated that random mutations introduced into T15VH CDR2 and framework region 2 frequently resulted in loss of binding or failure to be secreted (25, 26, 56, 57). However, group II V regions appear to be equally susceptible to harmful mutations (54, 56, 58). A caveat of these studies was that only limited regions of T15 and group II Abs were mutated in vitro. As identification of somatic mutation altering Ig secretion was a novel observation, we wanted to determine whether some of the mutations generated during an immune response in vivo would also be deleterious to Ab function. All mutated VH genes chosen for expression with the germline T15 L chain were impacted by somatic mutation: two mutants exhibited impaired secretion and two lost binding to PC protein. The fifth mutant and the clone that contained a novel D region displayed selective binding to PC protein-containing Ags with very little or no binding to PC displayed in the context of other carrier molecules. In addition to the secretion and binding loss mutants, four VH and one VL sequence contained stop codons. Two Vh and one VK, mutated within the V region genes, were likely introduced by somatic mutation in vivo. The two stop codons in the J gene of the clonally related sequences (G1.1 and G1.2, mouse 9) could have arisen via somatic mutation or during gene rearrangement. One clone (G4.2, mouse 8) had a deletion in the region of the internal heptamer resulting in stop codons, indicating perhaps an unsuccessful secondary rearrangement attempt. This sequence was obtained from a single T15 Id⁺ TUNEL⁺ cell (data not shown). These results suggest that somatic mutation in germinal centers and possibly secondary rearrangements may frequently lead to loss of binding, impaired Ig secretion, and potentially apoptosis. Overall, our minimum estimate of the magnitude of impairment of the in vivo-mutated Abs shown in Fig. 3 is 33% (9 of 27). If these cells die within germinal centers, such mutations may contribute to the high rate of apoptosis seen in germinal centers (59) and would be in agreement with models of B cell selection within germinal centers (60–64).

At present it is unclear whether B cells expressing nonfunctional Ig receptors can continue to proliferate before selection. Among our T15 H chain sequences, two sets of clonally related sequences are nonfunctional. Clonally related sequences G1.1 and G1.2 (mouse 9) both contain stop codons within the J region that would preclude Ab expression. In addition, clonally related sequences G4.19 and G4.23 (mouse 9) were secretion impaired when coexpressed with the germline T15 L chain. Although these data suggest that some proliferation may occur after deleterious mutation, alternative explanations are also possible. These VIH genes may belong to the excluded allele or have undergone receptor editing and now express a second functional H chain. In addition, the mutational status of the L chain originally paired with the secretion-defective H chains is unknown and might have compensated for the secretion defect. In most cases, we could not address the L chain mutation status as PCR amplification of both rearranged H and L chains from microdissected single cells proved difficult. Thus, it is formally possible that compensatory mutations in the L chain may have existed. Studies are currently underway to examine directly the proliferative potential and in vivo fate of B cells harboring deleterious Ig mutations and to determine whether compensatory mechanisms may rescue Ab function during an immune response as we have demonstrated in our in vitro studies (65–67).

Molecular basis of altered binding

We examined the molecular contributions of in vivo-generated mutations to PC binding and carrier recognition in a computer-generated model of the T15 combining site (38). Mutant VIH1-4 contains a single mutation located between the first and second loops of HCDR2. The Thr⁵⁶ to Ala replacement would disrupt both the Thr⁵⁶ H bond to Arg⁵² and to Asn⁵³ (Fig. 7A). These disruptions may influence binding in two ways: first, by increasing the conformational flexibility of the Arg⁵² side chain that directly contributes to PC binding via a critical salt bridge to PC (25, 38, 68); second, the substitution may alter carrier recognition (Fig. 6) either directly through the loss of the surface-exposed Thr⁵⁶ or indirectly by the disruption of the H bond formed with Asn⁵³ located in the first loop of HCDR2. These results underscore our previous observation that mutations in the first loop of CDR2 affect carrier recognition and discrimination among PC-containing Ags (35). The carrier discrimination of VIH1-2 is even more pronounced as it binds PC protein better than WT yet displays poor binding to S.
pneumoniae, T. spiralis, and A. suum. The binding profiles of mutants V1-4 and V1-2 emphasize the role of somatic mutation and selection in modification of the combining site toward specific recognition of the immunizing Ag.

Two mutants, V1-1 and V1-10, bind poorly to all of the five PC-containing Abs. V1-1-1 contains only two mutations. The Tyr91 side chain hydroxyl forms a hydrogen bond with PC and thus mutation of this contact residue would directly alter binding (Fig. 7B). The Glu to Asp change may also contribute to loss of binding as the residue at position 6 is a key amino acid in framework 1 confirmation, folding, and stability of single-chain Fvs (69, 70). Mutant V1-1-10 is more difficult to analyze as it contains three amino acid replacements and a D region insertion. Both the Ala126 to Thr and Asp202 to Ser mutations have been observed in anti-PC Abs (39) while the Glu36 to Gly has not been previously reported nor has the Asp108 D region insertion. The Asp insertion could affect the Trp106 contact with PC (38) or the Tyr106 interaction with L chain (65).

Molecular basis of altered secretion

Two clonally related V1H mutants containing either three (V1H-1-8) or four (V1H-1-6) amino acid substitutions failed to be secreted with the germline T15 L chain. This phenotype was consistent among multiple transfectants that each produced intracellular H and L chains but failed to readily form H\(\gamma\)L:\(\delta\) complexes (data not shown). In addition, mutant H chains displayed an association with molecular chaperones BiP and GRP94 implicating retention in the endoplasmic reticulum (data not shown), a result similar to our previous findings with Ab secretion mutants created by in vitro mutagenesis (27, 57). The molecular basis of secretion loss is difficult to dissect for mutants V1H-1-6 and V1H-1-8 because they contain multiple mutations. Both Abs contain an infrequent Tyr91 to His mutation. Tyr91 is part of a very conserved motif (YCARD) that is present in most Abs. Tyr91 is buried in the core of the VH and perhaps His91 cannot be accommodated within this buried region next to Cys92. Possibly this substitution disrupts the V1H domain disulfide bond. It is also possible that the Tyr106 to Asp mutation may be critical as Tyr106 directly contacts L chain. The removal of 4 amino acids (Ser99 to Trp106) rescued secretion in four T15 low secretion mutants indicating that HCDR3 conformation can alter secretion of some Ab mutants (65).

In summary, our data indicate that the failure of T15 Id+ Abs to maintain dominance in the late primary response to PC-KLH cannot solely be due to exclusion from the germinal center microenvironment or the somatic hypermutation process during the early primary response. When somatic mutations occurred, most of the T15 H chains tested by expression with a germline T15 L chain resulted in either impaired PC binding or nonsecretion. Mutations in HCDR2 and HCDR3 also significantly restricted Ab recognition to PC and PC protein and severely reduced or abrogated recognition of PC displayed in the context of different pathogenic microorganisms. In our model of repertoire shift, the high precursor frequency and B1 origin of T15+ B cells result in rapid Ab production in response to primary PC protein immunization. Some T15 cells enter germinal centers and are capable of undergoing somatic hypermutation. However, these cells are not expanded due to deleterious somatic mutation and clonal competition with group II Abs having more diverse combining sites that can mutate to bind the PC protein carrier with high affinity. Affinity based competition within germinal centers (45, 46) and the bone marrow (71) then leads to repertoire shift during the late primary and memory response.

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FIGURE 7. Computer generated model of the T15 combining site (38) showing the location of mutations in V1H-1-4 (A) and V1H-1-1 (B). A side view of the T15 Fv is displayed as a Ca trace with selected side chains. The V1H domain (green) is on the right and V1L (blue) is on the left. PC is shown in the combining site as a stick image. A, The location of Thr56 is shown as well as the PC contact residue Arg52 and Asn53. Shown are Thr56 side chain hydroxyl that forms a hydrogen bond with the O1 oxygen of PC. Atom colors: O is red; N is light blue, P is yellow, and C is white. The image was created with RasMac (version 2.6; MultiCHEM Facility, University of California, Berkeley, CA).
DELETEROUS SOMATIC MUTATIONS IN Ig V_{H}