POINT MUTATIONS CAUSE THE SOMATIC DIVERSIFICATION OF IgM AND IgG2a ANTIPHOSPHORYLCHOLINE ANTIBODIES

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During the past decade, it has become apparent that much of the sequence diversity of antibodies results from the combinatorial rearrangement of a series of small genetic elements (reviewed in reference 1) that together comprise the heavy and light chain variable (V) regions. Additional diversity is generated during the rearrangement of these genes through junctional diversity (1) and the generation of N sequences that are not present in the germline (2). Studies of families of mAbs derived from single germline V regions, and in some cases from one B cell precursor, have shown that somatic diversification (3) of already rearranged and expressed Ig V region genes significantly expands the antibody repertoire (3–9). The amino acid alterations that result from these diversification events can result in increases or decreases of antibody affinity (4, 5, 10) as well as changes in antigen specificity (6, 11).

Initially it was thought that hypermutation was solely responsible for somatic diversification of mAbs encoded by known germline genes (3–9). However, recently questions have been raised concerning other genetic mechanisms that may be involved in diversification (12–15), as well as the actual number of germline V region genes in the genome (16). These studies have implicated not only somatic point mutation but also V region replacement (12–14) and gene conversion (15) in Ig gene diversification. V region replacement has been demonstrated in vitro (12–14) and involves the replacement of the rearranged V region by an upstream V region. The second proposed mechanism, gene conversion occurs in vivo in chicken λ light chain where a series of pseudogenes donate sequences to a single rearranged V region gene (15) and has been suggested as the mechanism that generated a variable region mutation in vitro in a hybridoma cell line (17).

Although extensive studies of families of mAbs (3–9) suggest that sequence diversification arises through somatic point mutation, this has not been formally proven. In fact, since the base changes that are observed sometimes appear to
be clustered (8) and include substitutions of two or even three bases in a codon (5, 18–20), it is possible that either donor sequences or previously unidentified germline V region genes could account for the sequence differences found.

To directly address the issue of the genetic mechanisms involved in antibody diversity, we have examined two selected hybridomas which arose during the antiphosphorylcholine (PC) response in BALB/c mice. The response of BALB/c mice to PC, either associated with bacterial polysaccharides or coupled to protein carriers, has been extremely well studied (21). Almost all of the antibodies produced are encoded by the \( V_\alpha 1 \) gene of the S107 (T15) heavy chain V region family in association with any one of three light chains (21, 22). All of the T15 IgM anti-PC antibodies so far reported in the literature have the germline \( V_\alpha 1 \), DFL 16.1, and \( J_\alpha 1 \) sequences, while all of the IgG antibodies have numerous base substitutions (8, 19, 21). Mice that have been idiotypically suppressed (24, 25) or have genetic defects in their immune response (CBA/N) may express other heavy or light chain V regions (26).

We selected an IgM and an IgG2a anti-PC antibody for detailed study since they were likely to represent earlier and later stages in somatic diversification. Since most anti-PC IgMs have not undergone any somatic diversification (8), we screened 60 IgM anti-PC antibodies for loss of reactivity with a battery of anti-T15 antiidiotypic mAbs and identified one, P28, which had lost reactivity with a heavy chain–specific antibody (27). The IgG2a antibody, RP93, was isolated from a hyperimmunized mouse and had also lost reactivity with the same anti-heavy chain, antiidiotypic antibody, and in addition, no longer reacted with another antiidiotypic antibody that required the presence of the T15 light chain (27). The nucleic acid sequences of the heavy and light chain variable regions and the adjoining constant region domains were determined. Oligonucleotides specific for sequences that included the two base pair changes in the second hypervariable region of each of the heavy chains were then used to look for other V regions or potential donor sequences in the germline. Our results indicate that the base changes examined arose through somatic point mutation rather than through gene conversion, V region replacement, or the expression of previously undiscovered germline genes. We have also examined the impact of the sequence changes on the serology of the binding site.

Materials and Methods

Cell Lines and Cell Culture Conditions. The S107.3.4 (S107) myeloma cell line was originally obtained from the Salk Institute cell bank. The P28 hybridoma cell line was isolated from a BALB/c mouse initially immunized with 200 \( \mu \)g of PC conjugated to keyhole limpet hemocyanin (PC-KLH) emulsified in CFA (27). After 3 mo the mice were boosted with 200 \( \mu \)g of PC coupled to LPS and 3 d later the spleen cells were fused. The RP93 hybridoma cell line was isolated from a BALB/c mouse hyperimmunized with multiple injections of 250 \( \mu \)g PC-KLH emulsified in CFA and IFA. Two control PC binding mAbs were also used. PC-140 is an IgM T15 PC binding mAb that has been described previously (28). 8A1-G2a is an IgG2a PC-binding T15 mAb that was derived by in vitro heavy chain class switching from PC-223. W3129 is a dextran-binding IgA myeloma protein kindly provided by Dr. Sherie Morrison (Columbia University, New York, NY). All of the myeloma and hybridoma cell lines were maintained in an atmosphere
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of 8% CO₂ at 37°C in DME (Gibco Laboratories, Grand Island, NY) and supplemented with 10% FCS, 5% NCTC 109 (Gibco Laboratories), nonessential amino acids, glutamine, penicillin, and streptomycin (29).

Purification of S107, P28, PC-140, and RP93 Proteins. Ascites fluid containing hybridoma proteins was obtained by injecting 10⁷ exponentially growing cells into the peritoneal cavity of Pristane-primed (30) BALB/c mice. The hybridoma protein accumulated in the ascites fluid at 3–10 mg per ml. The PC binding proteins were purified by affinity chromatography over a PC-tyrosine-glycyl-Sepharose 4B column (31, 32). The bound antibodies were eluted with PC and the eluates were subsequently dialyzed to remove the hapten.

Serological Analysis. Direct binding ELISAs were carried out as described previously (33). The ELISA plates were initially coated with 10 µg/ml of purified PC-140, P28, or RP93 protein. After blocking with BSA, 50 µl of supernatants containing each of eight rat anti-T15 antibodies (27) were added. The amount of binding was detected using an alkaline phosphatase-linked goat anti-rat IgG (Zymed Laboratories, San Francisco, CA). The final optical density units were determined at 405 nm after a 1-h incubation at room temperature. For inhibition assays, ELISA plates were coated with 100 µl of 1 µg/ml purified S107 protein. The binding site-specific mAb, 4C11, was kindly provided by Dr. Heinz Kohler, Roswell Park, Buffalo, NY (34). Anti-PC binding site rabbit antiserum was prepared according to Claflin and Davie (31). An amount of anti-binding site antibody that bound 50% of the sites on the plate was incubated with increasing dilutions of inhibitor proteins, S107, P28, or RP93 for 1 h at room temperature. 50 µl/well of each dilution was added to the ELISA plates and incubated for 1 h at 37°C. The amount of bound 4C11 protein was determined using goat anti-mouse IgG (Zymed Laboratories) and the rabbit anti-binding site antibody was detected using enzyme-linked goat anti-rabbit IgG antisera (Zymed Laboratories).

Determination of Fine Specificity. ELISA plates were coated with 10 µg/ml PC-BSA. S107, P223, P28, and RP93 proteins were incubated with increasing concentrations of PC or one of two analogues, nitrophenylphosphorylcholine (NPPC) or choline (C), for 2 h at room temperature and then added to the PC-BSA-coated ELISA plate and incubated for an additional hour at 37°C. The assay was developed using class- or subclass-specific rabbit anti-mouse enzyme-linked antiserum (Zymed Laboratories) and the optical density units were determined.

Measurement of Affinity. The affinity for PC was determined using ultra-high speed centrifugation in an air-fuge (Beckman Instruments, Inc., Fullerton, CA) (35–37). 2 mg of purified protein were incubated with ¹⁴C-labeled PC (New England Nuclear, Boston, MA) in Tris/NaCl buffer at a molarity 10-fold less than that of the protein. 100 µl aliquots were added to six separate tubes containing increasing amounts of unlabeled PC and to one control tube containing no cold PC. The tubes were incubated at room temperature for 30 min and then 200 µl of each sample was centrifuged in 5 × 20 mm polycarbonate tubes for 3 h at 150,000 g (30 psi) at room temperature. The amount of bound ¹⁴C-PC was calculated from the amount of free ¹⁴C-PC.

Reverse Transcriptase Sequencing. Total RNA from S107, P28, and RP93 cells were extracted with guanidinium isothiocyanate and isolated over a CsCl gradient (38). Poly(A)⁺ mRNA was purified over an oligo-dT–cellulose column according to Maniatis et al. (39). 20 µg of Poly(A)⁺ mRNA was hybridized to labeled oligonucleotide specific for the region to be sequenced. Extension reactions were carried out according to Geleibter et al. (40) using reverse transcriptase in the presence of dNTPs and terminated with ddNTPs (41).

Probes and Primers. The location of each of the oligonucleotides used is indicated in the relevant figures. The S107 and P28 light chain V regions were sequenced using two oligonucleotides; a 20-bp primer (3' GGTGTCGACCTGAGCTTTTCAC 5') complementary to the S107A light chain 3' region of J₅, and a second 21-bp oligonucleotide (3' GCTATGTAACCACGGGACTA 5') specific for the middle of the V region. The RP93 light chain V region was sequenced with the following oligonucleotides: a 16-bp primer (3' GTAGAAGGGGTTGGATGG 5') specific for the 5' region of the C₅ domain, and a 20-
bp primer (3’ GCTAAAAGACCCCAGGGTCT 5’) specific for the middle of the RP93 V\_\_ region. The C\_\_ domains of all the antibodies were sequenced with the following two oligonucleotides: a 21-bp primer (3’ AAGTTGTCCTTACTCAGATTC 5’) specific for the 3’ end of the C\_\_ and a 21-bp primer (3’ AAGATGGGTTTTTCTGTAGTT 5’) specific for the middle of C\_\_.

The heavy chain CH1 domains were sequenced with the following oligonucleotides: IgM-specific; a 19-bp primer (3’ AGACGTACACGGGTAGTT 5’) complementary to the 3’ end of the CH1, a 20-bp primer (3’ TTAACCTACATTCAACGAGAC 5’) and middle primer (3’ AGGGGAGGAAAAGCGGTGCTAC 5’); IgG2a-specific 3’ end primer (3’ TGTGGTTCACCAGCTTCTTTTAA 5’) and middle primer (3’ TGGGAGGGTGAGCCAGGGgg 5’) and 5’ end (3’ CAGATAGGTGACCGG 5’). The heavy chain V region sequences were completed using the following oligonucleotides complementary to sequences in the middle of the V regions: RP93 (3’ ATTGTTATCTCATCAGG 5’); S107 (3’ GTGACACTTCCAGCGGAAGAG 5’); P28 (3’ GAAGACACTTCCACCCAGGAAAG 5’).

The oligonucleotides used as probes are specifically defined in the Results section. The s\^{}P nick-translated genomic probes were prepared from a 250-bp Dde I–Dde I fragment isolated from a plasmid containing a cDNA clone of S107 VH1 and a 2-kb Bam HI–Eco RI genomic probe containing the J3 and J4 genes and 3’ intervening sequence (42, 43).

**DNA Isolation and Gel Analysis.** High molecular weight DNA was isolated from the myeloma and hybridoma cells as described by Alt et al. (2). Approximately 10^8 cells were treated with proteinase K and extracted with phenol. The high molecular weight DNA was precipitated using cold 100% EtOH. Restriction enzyme digests were done and Southern blotting was performed by electrophoretically fractionating the DNA on an 0.8% agarose gel in 0.04 M Tris-acetate, 0.05 M NaOAc, 0.001 M EDTA. The DNA was transferred to Genescreen (New England Nuclear). Molecular hybridizations were done using procedures from Zeff et al. (44).

**Results**

**Identification of Variants.** Our goal was to study the mechanism and impact of somatic diversification both early and late in the immune response by examining T15’ anti-PC mAbs derived from PC-immunized BALB/c mice. To identify an mAb from early in the process of somatic diversification, 60 IgM anti-PC antibody–producing hybrids were screened using 8 different anti-T15 antibodies (27). Only one of the 60 IgM anti-PC–producing hybridomas, P28, was found to have altered serology when compared with the germline represented by the mAb PC-140 and the prototype S107 antibodies (27). In addition, one IgG2a anti-PC–producing hybridoma, RP93, from a hyperimmunized mouse also did not react with the same antidiotopic mAb. Fig. 1 illustrates the binding results of the P28, RP93, and PC-140 proteins using five of the eight anti-T15 antibodies. As can be seen, both the P28 and RP93 proteins do not react with the antidiotype antibody TC54 while PC-140 reacted with all of the antidiotypic antibodies. In addition to the loss of TC54 reactivity, the RP93 protein also did not react with a second antidiotype, TC139. It has been shown previously that the TC54 antibody is specific for the V region of the heavy chain (V\_\_\_) while the TC139 antibody requires the presence of both the T15 heavy and light chains (27). The results indicate that both the P28 and RP93 antibodies possess the dominant T15 heavy chain idiotype because they were recognized by the majority of the anti-T15 antibodies and because both antibodies bind PC. The loss of TC54 reactivity, however, suggested that both the P28 and RP93 antibodies had
undergone diversification of their V\textsubscript{H} regions, resulting in the loss of the idiotope. In addition, the loss of TC139 reactivity by the RP93 protein suggested that the T15 light chain was either not present or had undergone diversification in RP93.

**RNA Sequencing.** To determine the structural changes associated with the somatic diversification, the nucleic acid sequences of both the heavy and light chains of the P28 and RP93 antibodies were determined by reverse transcriptase sequencing of poly(A)\textsuperscript{+} RNA. The V\textsubscript{H}, D, J\textsubscript{H}, and the adjoining CH1 domains of the heavy chains and the V\textsubscript{\kappa}, J\textsubscript{\kappa}, and C\textsubscript{\kappa} domains of the light chains were sequenced (Figs. 2–4). The remaining domains of the heavy chain constant regions were not sequenced because SDS-PAGE gel analysis of both P28 and RP93 proteins did not reveal any major domain losses (data not shown). The sequences of the heavy and light chain of the P28 mRNA and the heavy chain of the RP93 mRNA were determined simultaneously with and compared with the sequence of the anti-PC antibody S107. This was done because the S107 antibody is known to be encoded by the rearranged T15 V\textsubscript{1} DFL 16.1 and JH1 germline genes and thus provided a germline control on each sequencing gel (45).

Fig. 2 shows the nucleic acid and translated amino acid heavy chain sequence of the IgM P28 antibody compared with the S107 antibody. There were two base pair differences in and just 3' to the second hypervariable (HV2) region and one in the J\textsubscript{\kappa} region (as shown by the bold lettering). The only exchange substitution was the one just 3' of the HV2 at residue 71 which resulted in an isoleucine being substituted by a phenylalalanine. None of these base changes appear in the other members of the S107 (T15) heavy chain variable region family or in any other published hybridomas derived from the V\textsubscript{1} gene (21). The CH1 domain did not differ from the IgM germline sequence (46, 47). The light chain V\textsubscript{\kappa}, J\textsubscript{\kappa}, and C\textsubscript{\kappa} regions of the P28 antibody were also sequenced and were identical to the V\textsubscript{\kappa}22 J\textsubscript{\kappa}5 germline sequence of the expressed S107A light chain (48) (data not shown). It was concluded that the P28 antibody had undergone somatic diversification and that the loss of TC54 reactivity by the P28 antibody was due solely to the altered amino acid at residue 71 in the V\textsubscript{\kappa} region.

The heavy and light chains of the IgG2a RP93 antibody were then sequenced to determine the degree of somatic diversification. The sequences of the heavy chain are shown in Fig. 3. There are 9 bp substitutions in the V\textsubscript{H} region of RP93.
when compared with the germline V<sub>h</sub>1 as expressed in S107. 6 of the 9 bp changes result in amino acid substitutions. In addition, RP93 has a junctional change in the 3′ codon of V<sub>h</sub>1, which also results in an amino acid change. None of these base changes are present in the germline genes of other members of the T15 VH gene family or in the published sequences of other relevant mAbs (21).

Starting with the base change and amino acid substitution at the VD junction, the sequences of S107 and RP93 diverge. RP93 does not use the same D as S107. The sequences between the V and J in RP93 do not correspond to a known D and could represent either an unreported D, N sequences, or some combination of these. In addition, RP93 uses J<sub>H4</sub> rather than J<sub>h1</sub> and has deleted the first 3 codons of the germline J<sub>H4</sub> sequence (Fig. 3) (43, 44). The remainder of J<sub>H4</sub> is germline in sequence and the C<sub>1</sub> domain of RP93 is identical to the
Figure 3. Nucleic acid sequence and translated amino acid sequence of the heavy chain V, D, J, and CH1 regions of the RP93 antibody compared with the S107 antibody. In the V region, the sequence homologies are indicated by the dashed lines and in the CH1 domain, the dashed lines represent identity with the germline constant region sequence. The base pair and amino acid differences are indicated in bold lettering. The oligonucleotide primers used for determining the nucleotide sequences are illustrated by the slashed boxes.

published IgG germline sequences of this domain (49). None of the base pair changes or amino acid substitutions in RP93 coincide with the changes found in the P28 heavy chain sequence so loss of binding to TC54 in RP93 must be due to a conformational change in the TC54 epitope.

The light chain of the RP93 antibody was also sequenced (Fig. 4) and it shared only 50% homology with the S107 VK region. However, the sequence was found to be highly homologous to the NH2-terminal 43 residues that had been reported for a T15 anti-PC antibody, 1B8E5, isolated from a CBA/N mouse (26). The homologous residues are indicated by the dashes. In addition to the nonhomologous VK regions, the RP93 light chain was found to use the JK1 region rather than the JK5, which is used by the S107A light chain (48). The CK domain, however, was found to be completely homologous with the germline sequence (50, 51). The lack of binding of TC139 antiidiotype antibody (Fig. 1), which requires the presence of the T15 light chain, is thus explained.
FIGURE 4. Nucleic acid sequence and translated amino acid sequence of the V, J, and C genes encoding the light chains of the RP93 antibody compared with the NH2-terminal amino acid sequence of the anti-PC antibody, IB8E5, isolated by Clarke and colleagues from a CBA/N mouse (26). The oligonucleotide primers used for determining the nucleotide sequence are indicated by the slashed boxes.

Identification of Genetic Mechanisms. DNA isolated from the P28, RP93, and S107 cell lines and BALB/c liver was examined with mutant and wild-type oligonucleotides and a variety of restriction enzymes in an attempt to determine the genetic mechanisms responsible for the base pair changes represented in both the P28 and RP93 antibodies. In Fig. 5, high molecular weight DNA from S107, P28, and BALB/c liver cells was digested with Eco RI and the fragments resolved on an 0.8% agarose gel. Fig. 5A shows a blot hybridized under stringent conditions with an oligonucleotide (Fig. 2, lower slashed bar over HV2) specific for the 2 bp changes in and around the HV2 region in the P28 heavy chain. We chose these two base pair changes because they were closely linked and therefore were good candidates for a donor sequence or an unidentified \( V_h \) gene. The results revealed a single 7.8-kb fragment present only in the P28 sample and not...
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FIGURE 5. Southern blot analysis of S107, P28, and BALB/c liver DNA samples. The DNA was digested with Eco RI and the fragments were resolved by agarose gel electrophoresis. The blot represented in A was hybridized under stringent conditions using a P28-specific oligonucleotide that included the two A-T substitutions in and just 3' to HV2 (Fig. 2). (B) The same blot stripped and rehybridized under stringent conditions with a T15 V_{
1} gene-specific genomic probe (see text for details). The 2.8- and 3.4-kb fragments always hybridize less well with this probe.

in either the S107 or genomic (liver) DNA samples. To ensure that the P28 specific oligonucleotide was recognizing the rearranged V_{
1} gene in addition to being P28 specific, the blot shown in A was stripped and rehybridized with a T15 V_{
1}-specific genome probe. In Fig. 5B, it can be seen that the probe identified the four major fragments reported by Crews et al. (45) that represent the four well-characterized members of the T15 V_{
1} gene family. It is known that the slowest migrating fragment (7.8 kb) represents the unrearranged T15 V_{
1} gene and that the migration of this fragment is not affected by VDJ rearrangement in the S107 cell line (45). Other crosshybridizing bands, such as the 3.6-kb fragment in the BALB/c liver sample, frequently appear in such blots (45). The fragment identified by the P28-specific oligonucleotide was found to comigrate with the expressed V_{
1} gene identified in the S107 sample. The presence of the hybridizing bands in the S107 and BALB/c liver DNA samples indicated that there was DNA present in all the lanes, thereby confirming that the P28-specific oligonucleotide was reacting only with P28 DNA.

Failure to identify any other hybridizing bands in the genomic DNA sample using the P28-specific oligonucleotide suggested the absence of any donor sequences or any P28-like germline V_{
1} gene; thus making it unlikely that gene conversion or V region replacement were mechanisms responsible for the base pair changes found in the P28 antibody. To further rule out these mechanisms, two additional sets of experiments were done. The first experiment dealt with the possibility that the Eco RI digestion might not reveal a potential donor
sequence and involved the digestion of the DNA with two additional restriction enzymes, Pst I and Hind III, and hybridization of the blots with the same probes described above (data not shown). In the second experiment, the DNA was digested with Eco RI and then hybridized to oligonucleotides synthesized to the individual base pair changes (upper slashed bars in Fig. 2) in and around the second hypervariable region on the chance that one of the base pair changes arose through somatic point mutation while the other was present in a potential donor sequence (data not shown). Both sets of experiments failed to reveal either a potential donor sequence or a P28-like germline gene, verifying the data shown in Fig. 5. Therefore, we concluded that somatic point mutation is the most likely mechanism responsible for the base pair changes in the P28 variant antibody.

Mutant-specific oligonucleotides were also used to examine the genetic mechanism responsible for the two base pair changes in the second hypervariable region of the RP93 heavy chain (see Fig. 3). Various restriction digests were probed with the 20-base oligonucleotide represented by the slashed bar in Fig. 3. The Pst I and Hind III digests are shown in Fig. 6. When DNA is digested with Pst I and Hind III and hybridized with the RP93-specific oligonucleotide under stringent conditions, only DNA from the RP93 cell line hybridized with the oligonucleotide, revealing a 3.2- (Pst I) and a 6.6-kb (Hind III) fragment, respectively (Fig. 6A). No hybridizing bands were found in the S107 or BALB/c liver DNA samples. The data suggest that, similar to the changes in the P28
antibody, the linked base pair changes in the HV2 region of the RP93 antibody most likely arose through somatic point mutation rather than from a donor or RP93-like Vn gene sequences in the genomic DNA. To confirm that the fragments identified in panel A also represented the rearranged and expressed T15 Vn1 gene, the blots were stripped and rehybridized with an oligonucleotide that was complementary to the unmutated sequences in the HV1 region of Vn1 in the S107, RP93, and BALB/c liver DNA. The results are shown in Fig. 6B, where it can be seen that the identical rearranged 3.2- and 6.6-kb bands seen in A also hybridized. Similar blots were hybridized with a Jn-specific genomic probe and confirmed that the 3.2- and 6.6-kb fragments represented the rearranged genes in S107 and RP93, respectively (data not shown). The differences in size between the hybridizing bands found in the S107 and RP93 samples (Fig. 6B) are fully accounted for by the location of the Pst I and Hind III sites located 3' to the Jn1 and Jn4 regions. The absence of an unarranged germline Vn1 in S107 is due to the deletion of the T15 family on the other allele, presumably during an aberrant rearrangement that preceded the myc into the a switch site. The lack of a germline rearrangement of Vn1 in RP93 is assumed to be due to segregation during the propagation of the hybridoma.

Since the substitution of A for G just 3' to the HV2 region (see Fig. 3) created a new Taq I site, we were able to also use Taq I digestion and hybridization with a genomic Vn1 probe (52) to confirm that the mutant gene encoding RP93 replaced the wild-type Vn1 gene, i.e., that the RP93 heavy chain was not encoded by a previously undescribed Vn1-like gene (data not shown).

Antigen Binding Analysis. It is generally accepted that during the course of the immune response somatic diversification and subsequent selection by antigen result in the expression of antibodies with progressively higher affinity. To examine the impact of the one amino acid substitution in the P28 antibody and the multiple changes and the unusual D, J, and light chain in the RP93 antibody, we compared the specificity and affinity of these two antibodies with the S107 antibody and with IgM and IgG antibodies that had not undergone somatic diversification. The specificity of the antibodies was initially compared by determining their ability to bind the putative natural antigen, pneumococcal C polysaccharide. The P28 antibody bound as well to the antigen as the germline-encoded IgM antibody. The binding of the RP93 antibody was compared with an IgG2a anti-PC antibody that had been generated in vitro by class switching from an IgM-producing hybridoma and is known to be encoded by the T15 heavy and light chain V regions (33). While the control IgG2a antibody had easily detectable binding to pneumococcal C polysaccharide at an antibody concentration of 1 µg/ml, RP93 did not show any binding even at 100 µg/ml.

The fine specificity of the P28 and RP93 antibodies was compared with that of S107 and a germline-encoded IgM antibody, P223, in an inhibition ELISA using PC and two of its analogues, NPPC and choline. The results using S107 and P223 protein represent the usual fine specificity patterns for germline T15 antibodies, i.e., PC inhibits more effectively than NPPC, which is more effective than choline (Fig. 7). The fine specificity pattern for the P28 protein is shown in Fig. 7C. It can be seen that the order of binding of PC and choline is similar to that found with the S107 and P223 proteins. NPPC, however, was barely
distinguishable from choline. This has been a reproducible finding. In contrast to the other antibodies, the RP93 antibody required less (10\(^{-5}\) M) NPPC for 50% inhibition, while the concentration at which PC inhibited was 10 times higher. The difference in binding is not due to the \(\gamma_2a\) constant region since in other studies (Spira, G., et al., manuscript submitted for publication) we have shown that P223 and an IgG2a antibody derived from it by in vitro switching have the same fine specificity. Therefore, RP93 clearly possesses an altered fine specificity.

Affinity data confirmed the above binding results. When P28, RP93, and S107 proteins were examined, their \(K_a\) values were as follows: S107 = 3.4 \(\times\) 10\(^5\)/M; P28 = 2.5 \(\times\) 10\(^5\)/M; and RP93 = 1.6 \(\times\) 10\(^5\)/M. The \(K_a\) value of S107 represents the germline affinity of T15 anti-PC antibodies. The \(K_a\) value of the RP93
antibody for PC was found to be lower than that of the S107 protein, while the value for the P28 antibody was in between. The affinity values for S107 have been determined in our laboratory on numerous occasions. The values were found to be in the range of $2.2-3.3 \times 10^5$/M, so we believe that the difference between S107 and RP93 represents a real decrease in affinity for PC. The lower affinity value of RP93 for PC coincides well with the fine specificity results, which show a preferred binding to NPPC rather than PC by the RP93 antibody.

**Binding Site Analysis.** The altered affinity and specificity of RP93, and perhaps of P28 antibodies suggested a change in the conformation of the binding site of both of the proteins. To confirm that the binding sites had undergone significant conformational changes, both proteins were tested with a binding site–specific mAb (4C11) and a binding site–specific polyclonal antiserum by inhibition ELISA. Fig. 8 shows the reactivity of S107, P28, and RP93 proteins with the antidiotype mAb 4C11. The 4C11 antibody was shown to be binding site specific under the conditions used because it was completely inhibitable by PC (indicated by the black box). Both the S107 and P28 proteins efficiently inhibited the binding of 4C11 to S107, while the RP93 protein did not. However, an ~60-fold higher concentration of P28 protein than S107 protein was required to inhibit, suggesting a difference in the binding sites of the P28 and S107 proteins. Similar results were obtained when the polyclonal anti–binding site–specific antiserum was used (data not shown).

These results, taken together with the fine specificity and affinity data, suggest that the binding site of the P28 antibody has undergone a minor conformational change due to the single amino acid substitution at residue 68, while the binding site of the RP93 antibody has undergone a major conformational change due to its amino acid substitutions, its unusual D, J, and light chain, or some combination of these.

**Discussion**

The analysis of large numbers of mAbs has led to the conclusion that point mutations are primarily responsible for the somatic diversification of already rearranged and expressed V region genes that occurs during the immune response (3–9, 18–21). Three recent findings, however, have raised important
questions about this interpretation of the results. Gene conversion has been shown to play a major role in creation of the λ light chain repertoire in chickens (15); V region replacement has been shown to occur in vitro (12-14); and data have again been presented suggesting that the germline V_h region repertoire may be much larger than had been predicted from other studies (16). These findings make it important to formally examine the genetic and molecular mechanisms responsible for the base changes that appear during the immune response. While it is not a simple matter to establish the mechanism responsible for each base change, we have attempted to do this for a few of the changes we have observed in two mAbs that are presumed to arise from the V_h1 gene of the S107 (T15) family. We picked this system because the S107 (T15) V_h region family is small and very well studied. Since it has been well documented that most T15 IgM antibodies have not undergone somatic diversification (8), we had to screen 60 such monoclonals with antiidiotypic antibodies to find one that appeared to have undergone somatic diversification. Based on its class, the fact that it is the first T15 IgM PC binding monoclonal to differ in its sequence from the germline, and the absence of base pair changes in the light chain, we have made the assumption that it represents an early stage of somatic diversification. We chose an IgG2a PC binding antibody expressing the V_h1 gene from a hyperimmunized animal to represent a later stage in somatic diversification. Since IgG2a antibodies containing the T15 (V_k22) light chain are not observed in vivo or found in mAbs (21, 53), it is not surprising that the RP93 antibody possesses a different light chain.

Although we cannot prove that these antibodies represent different stages in the immune response, their sequences and characteristics of the antibody suggest that this is true. For example, the larger number of base pair changes in the RP93 antibody is consistent with this assumption. Other properties of the RP93 antibody also suggest that it is a late product of the immune response. The animal from which it was derived had been hyperimmunized with PC-KLH. NPPC more closely represents the linkage group that results when PC is attached to a protein carrier and is probably a better reflection of the epitope that drove the secondary response than PC. This could explain why RP93 reacts better with NPPC than PC and in fact has a lower affinity for PC than S107. On the other hand, RP93 is still readily inhibitable by PC so it does not fall into the group II PC binding antibodies that arise during the secondary response (53).

If the base pair change differences we observed in both the P28 and RP93 antibodies were due to the expression of previously unidentified V_h1-like germline genes, gene conversion from an as-yet-unreported donor, or V region replacement, then closely linked base changes provide the best way to find the responsible genetic material. We have therefore used mutant-specific oligonucleotides to look for such sequences in the germline. Our inability to find such donor sequences strongly suggests to us that the P28 and RP93 antibodies were not derived from germline V region genes other than V_h1 either by the direct expression of such genes or through V region replacement.

It is more difficult to formally rule out gene conversion. It is possible that a donor sequence might have been either too large or too small to be detected in our gels. This seems unlikely since we have used a variety of restriction enzymes
to digest the liver DNA. It is also possible that one of the two base changes examined was present in the germline and the other arose through point mutation. We have ruled this out for P28 by examining each of the base changes individually. Finally, it is possible that the putative donor sequence is smaller than the oligonucleotide used and differs from it in its immediate flanking sequences. Since the P28-specific oligonucleotide we used goes beyond the base changes by only a few bases at each end (see Fig. 2), this also seems unlikely. Furthermore, the data in the literature suggest that gene conversion requires a donor of at least 24 nucleotides (54). However, since oligonucleotides of at least 15 bp, and in our case, often 18 or 20 bp, are required to obtain good hybridization, we cannot completely rule out multiple very small donor sequences acting sequentially. Nevertheless, we believe that the data presented here strongly support somatic point mutation as the genetic mechanism responsible for those base changes examined.

Each of the monoclonals examined was also inherently interesting. P28 is, to our knowledge, the first PC-binding IgM antibody reported to have undergone somatic mutation. It confirms the conclusions from other systems (4, 55) that class switching is not required for somatic diversification. It is also of interest to note that despite the many somatic mutations in the RP93 V region, there are no base changes in the C region. This result provides additional support for the widely held, though only moderately well documented (1, 7, 9, 18) view, that somatic mutation is restricted to the V region and its immediate flanking sequences in vivo. The small changes in affinity, fine specificity (similarity with NPPC and choline), and reactivity with antiidiotypic antibodies in P28 suggest that the single amino acid substitution not only caused the complete loss of reactivity with a non-binding site-specific antiidiotypic antibody (TC54) but also resulted in a minor but significant change in the structure of its binding site. This is consistent with the finding from three-dimensional studies of protein antigen-antibody interaction (56) that residues outside of the previously defined hypervariable regions may affect antigen binding. It is also worth noting that TC54 has been used in the literature (58, 57–59) to identify T15 antibodies and this shows that the epitope it reacts with can be lost while PC binding is retained.

While we had hoped that the P28 and RP93 antibodies would share some sequence differences and allow us to locate the TC54 epitope, this is clearly not possible since RP93 is a very unusual antibody. Despite the fact that RP93 uses the V\_n1 gene, still binds PC, and reacts with many of the anti-T15 mAbs, it differs from S107 and P28 by a number of amino acids in V and in the third complementarity determining region of the H chain. In addition, it uses a different heavy chain J region and contains a light chain that is very different from the V\_k22 light chain found in S107. Light chains of this same V\_k3 subgroup have also been reported (60) in type II PC binding antibodies that do not use V\_n1, are not inhibitable by PC (53) but are by NPPC, and do not express the T15 idiotype. Type II antibodies are frequently found in the secondary response to PC-KLH (53). In addition, a light chain that is very homologous to that of RP93 was also found in a PC binding antibody from a CBA/N mouse (26). CBA/N mice have an xid immunological deficiency gene that results in an
inability to make T15 anti-PC antibodies or to produce many other antipolysaccharide antibodies.

It is interesting to speculate on the origins of the RP93 antibody. It was derived from a mouse that had been repeatedly injected with PC-KLH in Freund's adjuvant. RP93 may have been derived from a B cell clone that originally did not react with PC on bacterial polysaccharides or perhaps even NPPC, but as a result of somatic diversification, acquired binding for both PC and NPPC and was then stimulated to proliferate and perhaps further diversify. While it is not possible to trace the origins of RP93, it does represent the kind of antibody discussed by Milstein and his colleagues (4, 18), which differ from those expressed early in the immune response and are brought into the immune response as it progresses.

Summary

The genetic mechanism responsible for the somatic diversification of two mAbs was determined. The two PC-binding hybridomas were representative of events early and late in the immune response. The P28 cell line that produces an IgM antibody and thus represents events early in the immune response, was found to have 3 bp changes in its heavy chain variable (V<sub>H</sub>) region, with some changes in antibody affinity or specificity. The RP93 cell line that produces an IgG2a antibody and thus represents later events in the immune response, was found to have 9 bp changes in its V<sub>H</sub> region resulting in decreased affinity for PC and altered specificity. Oligonucleotides specific for linked base changes in the second hypervariable regions of both of these antibodies were used to look for previously undescribed V regions or other donor sequences that could have been responsible for these base changes. Since no donor sequences were found, we have concluded that somatic point mutation rather than gene conversion, V region replacement or the expression of an unidentified germline V<sub>H</sub> region gene is truly responsible for at least some of the somatic diversification of these antibodies.

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