THE MONOCLONAL ANTI-PHOSPHORYLCHOLINE ANTIBODY RESPONSE IN SEVERAL MURINE STRAINS: GENETIC IMPLICATIONS OF A DIVERSE REPertoire

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The immune response to phosphorylcholine (PC) in BALB/c mice is dominated by a single antibody specificity (clonotype) which is identical to TEPC 15 (T15) and several other BALB/c myeloma proteins by the criteria of idiotypic cross-reaction and isoelectric focusing (1-5). Bone marrow-derived antibody-forming cell precursors (B cells) specific for PC have been found at a frequency of approximately 1/50,000 splenic B cells, of which 20-25% is not of the T15 clonotype (6).

The analysis of the response to PC as well as responses to other antigens has been greatly facilitated by the availability of antibody probes which are specific for immunoglobulin variable region idiotypic determinants. These anti-idiotypic sera have been used to investigate the mechanism of immune responsiveness (7, 8), the heterogeneity of the B-cell repertoire (3, 9-11), and the genetic information corresponding to immunoglobulin structure (12-14). In general, anti-idiotypic antibody is raised by immunization of syngeneic, allogeneic, or xenogeneic animals with a myeloma protein or a highly restricted antibody. After removal of antibodies which are directed against allotypic and constant region determinants of the immunoglobulins, the remaining antibodies have the ability to recognize variable region determinants. However, these antibodies are heterogeneous and may differ greatly in their recognition of sites (e.g., combining sites vs. framework regions) and in their ability to discriminate between antibodies which differ by only a few amino acids (11, 15).

Recently, two types of anti-idiotypic sera which are reactive against T15 have been developed. Immunization of A/He mice with T15 yields a murine anti-idiotypic antibody (M anti-T15) which recognizes anti-PC antibodies of mice that express the Ig-l" heavy chain allotype. The binding of M anti-T15 to T15 is minimally inhibited by hapten; thus, the anti-idiotype either binds the PC-combining site with a very high affinity (7) or is directed at variable region framework determinants outside the antibody-combining site...
In addition, Claflin and Davie (16) have described the preparation of anti-idiotypic antibody to HOPC 8, a protein idiotypically indistinguishable from T15, which is produced in rabbits and purified by adsorption to a HOPC 8 immunoadsorbent, followed by elution with PC. Since the binding of this anti-idiotypic antibody to HOPC 8 is completely inhibited by free hapten, it appears to be directed against determinants within the antigen-combining site. Rabbit anti-HOPC 8 was found to react with anti-PC antibody from several allotypically diverse murine strains. This observation, in combination with the M anti-T15 findings, implied that although the framework regions may vary between strains, the combining site itself may be highly conserved within the species (17).

We have prepared a rabbit anti-idiotypic antibody to T15 (R anti-T15) with hapten-modifiable properties similar to rabbit anti-HOPC 8. The availability of two anti-idiotypic antibody populations, M anti-T15 and R anti-T15, which recognize different portions of the variable region, has permitted a detailed analysis of the murine PC-specific repertoire. First, the findings indicate the existence of a heterogeneous population of B cells within the BALB/c PC-specific repertoire. Second, 2% of the detectable BALB/c PC-specific monoclonal antibodies reacted strongly with R anti-T15, but very weakly with M anti-T15. Third, the total frequency of cells specific for PC in A/He, AKR, and C3H mice was slightly lower than the BALB/c frequency. Fourth, although the three non-BALB/c strains produced monoclonal antibodies that were completely reactive with R anti-T15, such clones represented a minority of the nonimmune precursor cells; there does not appear to be a dominant clonotype expressed in the B cells of these strains. Furthermore, a minority of monoclonal antibodies which were derived from AKR B cells reacted as well with M anti-T15 and R anti-T15 as did BALB/c monoclonal antibodies. Thus, the use of anti-idiotypic reagents that can identify distinctive cross-reactivities within heterogeneous precursor cell repertoires has permitted the recognition and analysis of a variety of clonotypes which neither occur in unusually high frequency nor dominate the population of responding cells. The identification of similar combining sites on different variable region frameworks within the same strain and the sharing of similar combining sites and frameworks among murine strains are discussed in terms of the regulation of expression of the immunoglobulin variable region gene pool.

Materials and Methods

Animals and Antigens. 6-8-wk-old BALB/c AnN, A/He, C3H, and AKR mice were obtained from the Institute for Cancer Research, Philadelphia, Pa. AKR/J mice were also purchased from The Jackson Laboratory, Bar Harbor, Maine. New Zealand white rabbits were purchased from Skippack Farms, Skippack, Pa. The preparation of hemocyanin (Hy), phosphorylcholine-Hy (PC-Hy), and 3-(p-azophenylphosphorylcholine)-N-acetyl-L-tyrosyl-glycylglycine Boc hydrazide-hemocyanin (PPC-TGG-Hy) have been described previously (3, 6).

Plasmacytoma Proteins and Immunoadsorbents. The plasma cell tumors T15 and MOPC 460 were obtained from Dr. M. Potter, National Cancer Institute, National Institutes of Health, Bethesda, Md., and maintained by serial passage in BALB/c mice. Partially reduced and alkylated T15 ascites fluid was purified by acid elution from a PC-glycyltyrosine-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column (18), and MOPC 460 was purified by adsorption to 2,4-dinitrophenyl-lysine-Sepharose 4B and eluted with 0.5 N HCl (19). Purified MOPC 167, McPC 603, and MOPC 511 proteins, with specificity for PC, were the generous gift of Dr. Stuart Rudikoff, National Institutes of Health. T15, McPC 603, and MOPC 460 were conju-
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gated to Sepharose 4B by the procedure of Cuatrecasas (20). The preparation of PC-bovine serum albumin (BSA)-bromacetyl cellulose has been described previously (3).

Immunizations. BALB/c mice were immunized intraperitoneally with 0.1 mg of Hy in complete Freund’s adjuvant 2–3 mo before use as recipients in spleen cell transfer experiments. Mice to be used as immune donors received 0.1 mg of PC-Hy or PPC-TGG-Hy in complete Freund’s adjuvant and were used 2–4 mo later.

A/He mice were immunized with purified T15 or McPC 603 protein according to the protocol of Lieberman and Humphrey (21). Serum from individual mice which was specific for each idiotypic was used in this work. Two rabbits were immunized with 1 mg of T15 protein in complete Freund’s adjuvant in eight subcutaneous sites and 1 mo later with 1 mg of T15 in incomplete Freund’s adjuvant in the same sites. The rabbits were bled weekly and the injection and bleeding schedule was repeated 2 mo later. The sera from both rabbits showed similar patterns of hapten inhibition and reactivity with clonal antibody and thus were used interchangeably.

Purification of Anti-Idiotypic Sera. Anti-T15 sera or anti-McPC 603 sera from A/He mice were diluted 1:10 with 0.02 phosphate-0.15 M NaCl buffer solution, pH 7.4, and passed through a MOPC 410-Sepharose column. R anti-T15 which was specific for the antigen-combining site was prepared according to Clafiin and Davie (16). The serum was diluted 1:1 with 0.05 M borate buffer plus 0.15 M NaCl, pH 8.3, and passed through a column of T15-Sepharose. Then R anti-T15 was eluted with 10⁻⁵ M PC in borate-saline buffer, and repassed over the T15 column, which was equilibrated with 10⁻⁵ M PC. The eluate was extensively dialyzed against borate-buffered saline and then passed over a MOPC 603 Sepharose column, dialyzed, and concentrated by pervaporation.

Splenic Focus Technique. Spleen cell preparations from donor mice were prepared by teasing the cells out of the spleen into Dulbeco’s modified Eagle’s medium. The cells were 80–90% viable. 20 × 10⁸ cells from nonimmune donors and 5 × 10⁶ cells from immune donors were injected intravenously into syngeneic Hy-primed recipients that had been irradiated with 1,300 R 6 h earlier. Fragment cultures were prepared from spleens of recipient mice 16 h after cell transfer, as described previously (22). The fragments were individually stimulated in culture with PPC-TGG-Hy (5 × 10⁻⁷ M hapten), and culture fluids were changed every 2–3 days. Fluids collected 10–15 days after stimulation were assayed for anti-hapten antibody, heavy chain isotype, and idiotype.

Radioimmunoassays for Antibody and Idiotype. 20 μl of culture fluid was added to PC-BSA-bromacetyl cellulose and bound anti-hapten antibody was detected by the subsequent binding of ¹²⁵I-labeled purified rabbit anti-mouse Fab antibody or ¹²⁵I-labeled purified goat antibody against mouse μ-γ1- or α-heavy chain constant region determinants (3). The amount of antibody was quantified in the anti-Fab assay using T15 protein as a standard. T15, McPC 603, MOPC 167, and MOPC 511 all produced similar binding curves with the immunoadsorbent.

Idiotypic assays were performed by initially determining the dilution of anti-idiotypic antibody that produced maximal control binding and inhibition. The M anti-T15 sera used in this study were diluted 1:6,000 in phosphate-saline buffer and the anti-McPC 603 sera were diluted 1:3,000. Binding of T15 by purified R anti-T15 antibody was linearly related to the dilution made. Generally, a dilution that bound 10% of the added ¹²⁵I-labeled T15 protein was used with the addition of 0.017% normal rabbit serum. 1 ml of the appropriate dilution of anti-idiotype was added to 10 × 76-mm polystyrene test tubes (Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) and incubated for 4 h at room temperature. The serum was withdrawn and the tubes were washed with 1 ml of phosphate-buffered saline. Then 1 ml of phosphate buffer containing 1% BSA was added, and the tubes were incubated for 30 min at room temperature. The solution was removed and 20 μl of various concentrations of unlabeled inhibitor proteins or culture fluids was added, followed by the addition of 1.1 ml of 1% BSA in phosphate-buffered saline containing 2 ng of ¹²⁵I-labeled T15 protein, with a specific activity of 15–20 μCi/μg protein (23). After incubation at 37°C for 18 h, the tubes were washed three times with phosphate-buffered saline and counted in a gamma counter.

Hapten and Antigen Inhibition. In the radioimmunoassay for antibody, solutions of the hapten, PC in 0.15 M NaCl (saline), or the antigen, PC-Hy, were preincubated with myeloma protein or culture fluids for 1 h at room temperature. The mixture was then added to PC-BSA-bromacetyl cellulose such that the final concentration of free hapten or the haptenic determinant on the carrier was as specified in Table I. After 1 h, the tubes were centrifuged and washed; then ¹²⁵I-labeled anti-Fab was added to the pellet, and the procedure was continued as described previously (23). In the assay for idiotype, hapten or antigen was preincubated with ¹²⁵I-labeled T15 for 1 h and
TABLE I

Specificity of Anti-Idiotypic Antibodies

| Inhibitor* | Inhibition of $^{125}$I-labeled T15 binding to M anti-T15 | Inhibition of $^{125}$I-labeled McPC 603 binding to anti-McPC 603 |
|------------|----------------------------------------------------------|------------------------------------------------------------------|
| T15        |                                                         |                                                                  |
| 5 ng       | %                                                       | %                                                                |
| 50 ng      | 44                                                     | 66                                                               |
| McPC 603   | 50 ng                                                  | 0                                                                |
| MOPC 167   | 50 ng                                                  | 0                                                                |
| 10$^{-3}$ M PC | 0                                                              | 77                                                               |
| 10$^{-6}$ M PC-Hy | 57                                                      | 73                                                               |

* Unlabeled protein, hapten, or antigen was added as an inhibitor in the binding of 2 ng of $^{125}$I-labeled idiotype to anti-idiotype-coated test tubes.

Results

Specificity of Anti-Idiotypic Antibodies. Both M anti-T15 and R anti-T15 were specifically inhibited from binding $^{125}$I-labeled T15 by unlabeled T15, but not by two other PC-binding myeloma proteins, McPC 603 and MOPC 167. As seen in Table I, the M anti-T15 used in this study was not inhibited from binding T15 in the presence of free hapten, although PC-Hy was an effective inhibitor. R anti-T15, on the other hand, was effectively blocked from binding T15 by PC. Although both anti-idiotypes are heterogeneous antibody mixtures, the hapten inhibition data indicates that a majority of antibody in the R anti-T15 preparation can be generally classified as antigen-combining site-specific, whereas M anti-T15 may contain primarily antibody either with an affinity too high to be inhibited by hapten or directed against variable region determinants surrounding, but not in, the hapten-combining site.

In addition, the specificity of A/He anti-McPC 603 serum is shown in Table I. The anti-idiotype is specific for McPC 603, and the reaction is inhibited to a greater extent with PC-Hy than with the free hapten.

Anti-PC Frequency in Murine Strains. The frequency of B cells specific for PC from nonimmune and immune donors representing four murine strains was calculated using donor-cell homing and stimulation efficiencies previously described for BALB/c mice (24) (Table II). We have previously shown that the in vitro anti-PC response is maximized by using PC linked via a tripeptide spacer to the carrier (3). The overall frequency of cells specific for PC is only slightly lower in AKR, C3H, and A/He mice than that previously reported for the BALB/c strain (6). However, the total frequency of precursors responsive to PC (1 in 43,000–120,000 nonimmune B cells) is much lower than the frequency of cells that are specific for dinitrophenyl (1 in 5,000 cells) (22). Immunization with PC-Hy increased the frequency severalfold, which suggests the potential for the generation of a memory cell pool to even an ubiquitous antigen.
### Table II

**Frequency of PC-Specific B Cells in Nonimmune and Immune Mice**

| Strain | Number of clones/10^6 B cells* |
|--------|-------------------------------|
| BALB/c | 23.2 91.7                     |
| AKR    | 9.1  49.2                     |
| A/He   | 8.5  52.7                     |
| C3H    | 12.5 18.6                     |

* 276-924 × 10^6 cells from 11 to 23 nonimmune donor mice were subjected to clonal analysis, and 50-720 × 10^8 cells from 4 to 14 immune mice were examined.

**Characterization of Clonotypes.** Monoclonal anti-PC antibodies derived from B cells of the four mouse strains were analyzed for the presence or absence of M anti-T15 and R anti-T15 reactivity. A description of the major clonotypes that are delineated by the two anti-T15 sera is given in Table III. In BALB/c mice, four types of antibodies were distinguished: (a) monoclonal antibodies quantitatively reactive with both R anti-T15 and M anti-T15 (R T15+, M T15+), representing the dominant, i.e., T15, clonotype identified previously (6); (b) monoclonal antibodies not reactive with either M anti-T15 or R anti-T15 (R T15-, M T15-); (c) antibodies partially reactive in varying degrees with both anti-idiotypes (R T15±, M T15±); and (d) monoclonal antibodies significantly more reactive with R anti-T15 than with M anti-T15 on a weight basis. Antibodies in this latter group were divided into two subgroups: those reactive with R anti-T15 but not M anti-T15 (R T15+, M T15-), and those completely reactive with R anti-T15, when compared to Fab content, but only partially reactive with M anti-T15 (R T15+, M T15±). The cross-reactivity found with some antibodies in the latter group with M anti-T15 may be due to the presence in M anti-T15 of a minor fraction of antibodies with specificity for the antigen-binding site, or alternatively, may reflect cross-reactivity of some framework determinants.

In AKR mice, one group of clones reacted equally well when assayed by anti-Fab, M anti-T15, and R anti-T15, and thus was indistinguishable by these criteria from the R T15+, M T15+ clonotype in the BALB/c strain. Another group of AKR monoclonal antibodies did not react with either anti-idiotypic antisera, and a third group reacted partially with both antisera. A/He and C3H B cells produced monoclonal antibodies which were either: (a) equally reactive with anti-Fab and R anti-T15 but not with M anti-T15, (R T15+, M T15-), (b) nonreactive with both anti-idiotypic antisera (R T15-, M T15-), or (c) partially reactive with either or both antisera (R T15±, M T15±).

**Analysis of BALB/c Clonotype Heterogeneity.** An analysis of the reactivity of approximately 700 monoclonal antibodies from nonimmune and immune BALB/c donors with anti-Fab, M anti-T15, and R anti-T15 has been schematically represented in Fig. 1a and 1b as the ratio of the quantity of antibody detected with anti-Fab vs. the amount of antibody detected with M anti-T15 or R anti-
TABLE III

Idiotype Identification of Anti-PC Clones in Various Strains

| Strain   | Idiotypic identification | Nanograms detected by* |
|----------|--------------------------|------------------------|
|          |                          | Anti-Fab | R anti-T15 | M anti-T15 |
| BALB/c   | R T15+, M T15+           | 4.5       | 5.6       | 5.4       |
|          | R T15-, M T15-           | 10.0      | 0         | 0         |
|          | R T15±, M T15±           | 8.0       | 4.0       | 3.0       |
|          | R T15+, M T15±           | 4.8       | 2.7       | 0         |
|          | R T15+, M T15±           | 5.2       | 5.0       | 1.6       |
| AKR      | R T15+, M T15+           | 12.4      | 13.0      | 15.5      |
|          | R T15-, M T15-           | 19.0      | 0         | 0         |
|          | R T15±, M T15±           | 8.0       | 3.3       | 4.0       |
| A/He     | R T15+, M T15-           | 12.0      | 10.5      | 0.9       |
|          | R T15-, M T15-           | 20.0      | 0.2       | 0.1       |
|          | R T15±, M T15±           | 8.4       | 4.9       | 3.8       |
| C3H      | R T15+, M T15-           | 1.0       | 1.1       | 0.2       |
|          | R T15-, M T15-           | 8.6       | 0.8       | 0.6       |
|          | R T15±, M T15±           | 5.6       | 2.4       | 1.2       |

* The data represent assays of representative monoclonal antibodies with the various detecting reagents that were quantified by comparison with T15 protein.

T15. The majority of monoclonal antibodies fell into two areas, the T15-negative clones in region A and the T15-positive clones in region C.

In most of the experiments reported here, limiting dilution cell doses yielded between 5 and 20% positive fragments. Since homing of the donor cells follows a Poisson distribution (25), a small percentage of the positive fragments from recipient mice may contain more than a single precursor cell. The amount of antibody detected with anti-Fab would be greater than that detected with anti-idiotype if one of the precursor cells produced the T15 idiotype whereas the second precursor cell in the fragment did not. The expected number of such double fragments, i.e. predicted overlap, can be calculated by multiplying the frequency of foci which produce the T15 idiotype by the frequency of foci which produce non-T15 antibody (see reference 26 for the validation of independent precursor distribution in fragments). Foci producing partially idiotypic reactive antibody were placed in both categories since these fragments may contain both T15-positive and negative precursors. Therefore, the predicted overlap of 17 in Fig. 1a was derived by multiplying the total number of fragments by the product of the frequency of clones reactive with M anti-T15 (327 + 71 foci in 8,784 fragments) times the frequency of foci with partial or no T15 reactivity (311 + 71 in 8,784 fragments). This calculated number presumably represents the number of fragments that may contain more than one randomly lodged precursor cell.

In Fig. 1a and b, the actual number of clones that experimentally fall in region B (71 and 75) is seen to be greater than the predicted overlap frequency (17 and 18), which indicates that some partially reactive antibodies have arisen
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FIG. 1. Classification of BALB/c anti-PC clones from nonimmune and immune donors by Fab/idiotype ratios. Fig. 1a represents data plotted according to the ratio of nanograms of antibody detected by anti-Fab vs. M anti-T15; 1b represents data determined as the ratio of antibody detected with anti-Fab compared to R anti-T15. Monoclonal antibodies in region A had a ratio of Fab/idiotype of ≥5/1 (idiotype negative); those in region B were plotted as indicated; antibodies in region C had a ratio of 1.4/1 or less (idiotype positive). The experimental number of points in group B are noted. The number of points in region B that was predicted as the expected overlap in a fragment of two clones, one T15+ and the other T15−, was calculated by multiplying the frequency of total foci containing the T15 idiotype plus partially reactive antibody (C + B) by the frequency of foci producing non-T15 antibody plus partially reactive antibody (A + B). The product of the frequencies was multiplied by the total number of fragments to yield the number of fragments that may contain more than one anti-PC clone.

From single B cells and constitute a subset of clonotypes. Partial cross-reactions could be explained by the heterogeneous nature of the anti-idiotypic antibody. For example, if the anti-idiotypic contains three types of antibody directed to three theoretical determinants on the T15 molecule, a clone producing homogeneous antibody with one such idiotypic determinant would compete one-third as well with 125I-labeled T15 molecules for the anti-idiotypic sites in the inhibition assay. Therefore, the presence of partially cross-reactive antibody in low frequency experiments where biclonal fragments are not expected to occur may serve as a means of identifying new clonotypes.

The similarity of patterns in Fig. 1a and b suggest that both R T15 and M T15 idiotypic determinants are either absent, present, or partially present on the
same clonal product. Thus the ratio of antibody detected with R anti-T15 vs. the amount of antibody detected with M anti-T15 should be 1 if the idiotypic determinants are equally represented on the same molecule. As shown in Fig. 2, the majority of T15-positive clones and partially positive clones reacted equally well with both anti-idiotypes. However, a minority of clones non-reciprocally expressed the idiotypic determinants which were detected by R anti-T15 and M anti-T15. Thus, the R anti-T15/M anti-T15 ratio was significantly greater than one for 13 clones which showed reproducibly higher reactivity with R anti-T15 than with M anti-T15 on a weight basis (Fig. 2). The bimodal distribution reflects two subsets of clones which are described in Table II, that is, some of the clones (upper half of Fig. 2) reacted equally well with anti-Fab and R anti-T15 but had no reaction with M anti-T15 (R T15+, M T15−), and others (lower half of Fig. 2) were completely reactive with anti-Fab and R anti-T15 and had partial reactivity for M anti-T15 (R T15+, M T15±). This implies that the R T15 determinant(s) which is specific for the binding-site region can be present on some antibody molecules independently of the M T15 determinants.

The heterogeneity of the BALB/c PC-specific repertoire was further analyzed by the inhibition of the binding of monoclonal antibodies to PC-BSA-bromoace-
tetyl cellulose with $7 \times 10^{-4}$ M PC. This technique was used both as a very crude estimate of antibody-binding affinity as well as a way to further delineate subsets of clonotypes. Although binding of the R T15+, M T15+ antibody from BALB/c mice as well as T15 myeloma protein was completely inhibited at this concentration, the T15-negative foci appeared heterogeneous in their binding properties (Fig. 3), representing both high and low affinity antibody. Thus, by both hapten inhibition and anti-idiotypic reactivity, the T15-negative BALB/c antibodies probably represent a heterogeneous array of clonotypes.

A third approach to assessing the heterogeneity of the BALB/c PC-specific repertoire utilized an analysis of 141 T15-negative foci for the presence of antibody reactive with anti-McPC 603 antiserum. No monoclonal antibody was found that was reactive with anti-McPC 603 and anti-Fab on a 1:1 weight basis, but two foci were detected which exhibited partial (10-20%) cross-reaction with this anti-idiotypic. The finding that less than 2% of the non-T15 clonotypes share this specificity is a further reflection of the heterogeneity of the non-T15 PC-specific B-cell repertoire.

**Analysis of Clonotype Heterogeneity in Other Strains.** PC-specific monoclonal antibodies from nonimmune and immune A/He, AKR, and C3H donors were assayed with anti-Fab and the two anti-idiotypic reagents. In Table IV, the data is expressed as the ratio of nanograms of antibody detected with anti-Fab vs. nanograms of antibody detected with R anti-T15 or M anti-T15, where a ratio of $\geq 5/1$ is considered idiotypic negative and $\leq 1.4/1$ is idiotypic positive. All three strains have a significant percentage of antibody that is as reactive with R anti-T15 as with anti-Fab, as well as clones that do not have this idiotypic determinant. The hapten inhibition data in Fig. 3 again demonstrates the heterogeneous nature of this group of T15-negative clones in AKR, A/He, and C3H strains.

In all instances, the number of R T15 idiotype cross-reactive clones exceeds the predicted number of fragments that could contain two precursors, one idiotypic positive and one idiotypic negative. Clones partially reactive with M anti-T15 are also observed in a higher frequency than that predicted by the number of fragments which could contain two clones. These antibodies exhibited partial reactivity with R anti-T15 as well. While it is difficult to interpret the relationship between idiotypic cross-reactivity and antibody structure, the partially reactive antibodies from AKR, A/He, C3H, and BALB/c mice seem to be the clonal product of single precursor cells and tentatively can be identified by patterns of cross-reactivity. Variable region genes coding for cross-reactive idiotypic determinants on antibodies have been reported and genetically mapped by others (10).

21 foci from AKR mice were found that reacted equally well with anti-Fab and M anti-T15 on a weight basis. Antibody from each of these clones also reacted equally well with R anti-T15 as with anti-Fab, and by these criteria of variable region idiotypic expression were indistinguishable from T15. This particular clonotype was absent from A/He and C3H nonimmune and immune precursor cells.

**Frequency Analysis of Identifiable Clonotypes.** Table V lists the frequency of PC-specific B cells from nonimmune donors in each subpopulation that is
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Fig. 3. Hapten inhibition of binding of murine monoclonal antibodies to PC-BSA-bromoacetyl cellulose. Antibody previously determined to be either idiotype positive by both R anti-T15 and M anti-T15 (R T15+, M T15+) or negative (R T15-, M T15-) was tested for inhibition of its binding to PC-BSA-bromoacetyl cellulose by $7 \times 10^{-5}$ M PC in the radioimmunoassay. ○, purified T15 protein; ●, individual monoclonal antibodies.

**Table IV**

**Analysis of Murine Anti-PC Monoclonal Antibodies by Fab and Idiotypic Reactivity**

| Strain | ng Fab/ng R T15 | ng Fab/ng M T15 |
|--------|-----------------|-----------------|
|        | ≥5 | 5-1.4 | ≤1.4 | ≥5 | 5-1.4 | ≤1.4 |
| AKR    | 36 | 17    | 21   | 41 | 12    | 21   |
| A/He   | 42 | 11    | 10   | 59 | 4     | 0    |
| C3H    | 21 | 5     | 2    | 27 | 1     | 0    |

* Antibody from nonimmune and immune donors is expressed as the ratio of Fab/idiotype by weight. Values greater or equal to 5/1 are classified as idiotype negative, and ratios of 1.4/1 or less are termed idiotype positive. The number of partially reactive clones with antibody that exhibited some, but not complete, activity in anti-idiotype assays is indicated.
defined by R anti-T15 and M anti-T15 idiotypic analyses. Keeping in mind the hapten inhibition data presented in Fig. 3, these results imply a diverse array of specificities within the PC-specific repertoire since many unique clonotypes may exist within each subpopulation. For example, the R T15-, M T15- group most likely represents many clonotypes with idiotypic determinants other than T15. Table V also demonstrates that murine strains other than BALB/c do not possess a dominant antibody specificity such as the R T15+, M T15+ clonotype, which constitutes 75% of the BALB/c response. In fact, the corresponding clonotype within the AKR repertoire is present in a lower frequency, which suggests that genetic factors may be responsible for the clonal dominance of the T15 clone in BALB/c rather than affinity of the antibody for antigen. In A/He and C3H mice, the R T15+, M T15- clonotype represents a minor proportion of the PC-specific repertoire, although antibody with these characteristics has been reported by others to predominate the sera of mice immunized with pneumococcus containing the PC determinant (17).

Discussion

Intense interest in the analysis of the immunoglobulin variable region has led to widespread use of anti-idiotypic sera as genetic markers for this region. It is increasingly clear, however, that not all antisera recognize the same determinants within the variable region, nor do they necessarily discriminate among antibody molecules which have similar but not identical amino acid sequences. For example, an anti-idiotypic system has been defined by Claflin and Davie (27) which shows cross-reactions among PC-binding myeloma proteins with widely differing amino acid sequences. On the other hand, Hart et al. (9) have prepared anti-idiotypic sera which can discriminate between anti-azobenzenearsenate antibodies of individual mice within the same strain.

Since anti-idiotypic sera can recognize a variety of determinants and can vary in their ability to discriminate between closely related clonotypes, every antiserum must be defined within the context of the experimental system under study. This is particularly relevant when an analysis of idiotypic relationships of serum antibodies among strains is undertaken. The detection of low levels of an idioype in serum antibody may indicate weak cross-reactions among a diverse
population of antibodies, none of which are identical to the idiotype in question, or alternatively, the relevant idiotype may be present but represent only a small proportion of the serum antibody (28). The analysis of homogeneous antibodies generated in the splenic focus system provides an unambiguous determination of the presence or absence of a given clonotype and its representation within the responding B-cell pool. In addition, the partial reactivity of anti-idiotypic antibodies with a monoclonal antibody permits the identification of new clonotypes and has allowed us to uncover interesting relationships among anti-PC antibodies of BALB/c and other murine strains. Under conditions of individual B-cell stimulation in a milieu of excess carrier-primed T cells, we have previously determined the frequency of the dominant clonotype in the BALB/c PC-specific response as about 1/60,000 B cells and demonstrated the existence of non-T15 idiotype producing PC-specific clones (3, 6). This report extends the characterization of M anti-T15-positive clones by demonstrating that (a) all BALB/c monoclonal antibodies possessing the M anti-T15 idiotypic determinant express the R anti-T15 idiotype as well, and (b) T15 idiotype-positive monoclonal antibodies have similar hapten-binding (inhibition) properties. These characteristics, in addition to the isoelectric focusing pattern shared by T15 idiotype-positive monoclonal antibodies (4), indicate that the precursors of these clones belong to a single clonotype.

In BALB/c mice, it was possible to demonstrate a heterogeneous array of PC-specific clonotypes by anti-idiotypic reactivity and hapten inhibition of antibody binding to antigen. For example, the R anti-T15 assay has identified a subset of specificities that are not as reactive with M anti-T15 as with R anti-T15 on a weight basis. These antibodies apparently represent several clonotypes since they display heterogeneity by degree of cross-reactivity with M anti-T15 (Fig. 2). A maximum estimate of the frequency of this entire subset can be calculated to be one in $2 \times 10^6$ B cells, or 30-40 times fewer than the R T15+, M T15+ clonotype which is represented by 1 in every 60,000 B cells. Another example of idiotypic identification of low frequency clonotypes in BALB/c mice is demonstrated by the use of anti-McPC 603 raised in A/He mice. In an analysis of 141 monoclonal antibodies, two foci were found that partially cross-reacted with anti-McPC 603. While this result indicates that there were no PC-specific clones reacting on a 1:1 basis with both anti-McPC 603 and anti-Fab, a new clonotype subset is operationally defined on the basis of this cross-reaction. The frequency of this subset is approximately 1-2 in $10^7$ B cells. Furthermore, hapten inhibition studies have revealed a wide range of affinities among non-T15 antibodies, which suggest the existence of many distinct antigen-combining sites for PC. Thus, by idiotypic analysis and hapten inhibition, the BALB/c PC-specific repertoire consists of a single predominant clonotype and a diverse array of others.

Dissection of the PC-specific repertoire of other murine strains with R anti-T15 and M anti-T15 reveals that no strain other than BALB/c has a single predominant clonotype. The frequency of PC-specific B cells in AKR, A/He, and C3H mice is about 1 in 100,000 B cells compared to 1 in 50,000 in BALB/c. Although the repertoire in these strains is more difficult to define precisely, it is clearly heterogeneous by both hapten inhibition analysis and idiotypic cross-
reactions. Some monoclonal antibodies were recognized by neither anti-idiotypic, some cross-reacted weakly with both R anti-T15 and M anti-T15, and some were identified as R T15+ and M T15−. The R T15−, M T15−, and R T15±, M T15± subsets each apparently represent several clonotypes since antibodies in the former group show diversity in their affinity for hapten, and those in the latter group vary in their reactivity with both anti-idiotypic preparations. The low levels of M anti-T15 reactivity seen by others (13) in the serum of A/He and C3H mice were apparently due to weak cross-reactions among a diverse antibody population rather than to the presence of a low frequency of molecules idiotypically identical to T15.

Serum antibody from AKR, A/He, and C3H mice had been reported to be homogeneous by binding characteristics and complete reactivity with R anti-T15 but not with M anti-T15 (17), although more recent work suggested that several more clones are involved (5). Monoclonal antibodies with these characteristics (R T15+, M T15−) were found at a frequency of 1/500,000 B cells in A/He and C3H mice; however, this clonotype represented only 20% of the nonimmune B cells specific for PC, with the other 80% appearing quite diverse. Thus, under conditions of individual B-cell stimulation in the splenic focus system with a hapten-carrier complex which maximizes T-cell participation in stimulation, a diverse array of specificities is stimulated. The existence of a heterogeneous array of non-T15 idiotype-producing cells in BALB/c mice has been revealed by the same techniques, emphasizing the value of this assay in detecting the majority of cells capable of being stimulated by a given antigen (3).

The T15 clonotype in BALB/c mice is unique compared to other clonotypes which are specific for PC in other murine stains. The frequency of the R T15+, M T15− subset in AKR, A/He, and C3H is at least 10-fold lower than the predominant clonotype in BALB/c. Furthermore, in the specificity repertoires of B cells responsive to a variety of determinants including dinitrophenyl (29), 4-hydroxy-5-iodo-3-nitrophenacetyl (30), and PC, the T15 clonotype in BALB/c mice is the only clonotype identified with such a high frequency. This observation implies that the frequency of the T15 clonotype may not be representative of the entire repertoire. Whatever mechanism is responsible for the unusually high representation of this clonotype in BALB/c mice, it is unlikely that antigen contact plays a role in the expansion of the T15 clonotype, since the frequency is the same in germfree and conventionally reared donors (6).

In AKR mice, a group of clones reacts equally well with anti-Fab, R anti-T15, and M anti-T15. These monoclonal antibodies would thus appear to be idiotypically indistinguishable from the T15 clonotype in the BALB/c strain. Previous genetic studies have failed to reveal the presence of the M T15 idiotype in AKR mice (13, 17, 31). Although Table I demonstrates that the M anti-T15 serum used in this study is similar by several criteria to that used by other investigators, a possible explanation for the detection of T15-positive clones in AKR mice is that this antiserum has some properties which differ from those defined previously. It should be noted that preliminary analysis indicates cross-reactivity of this M anti-T15 serum with the MOPC 511 PC-specific myeloma protein, a finding also reported by Lieberman and Potter for their M anti-T15 sera (32). Nevertheless, a strong relationship exists between the AKR and BALB/c specificities. Whether
this idiotypic similarity is due to the complete sharing of variable region genes between these allotypically distinct strains will only be discerned by the amino acid sequence analysis of the AKR monoclonal antibody.

Since the AKR T15-positive clonotype is found in every individual of the strain, it presumably reflects a germ line antibody specificity. However, the frequency of the AKR clonotype is 2.7/10^6 B cells, or sixfold lower than the frequency of the idiotypically similar BALB/c T15 clonotype, implying that not all specificities represented in the germ line need be expressed at a high frequency. The mechanism responsible for the differential expression of similar antibody specificities in allotypically distinct strains may lead to an understanding of the role of regulatory genes in variable region expression and of the phenomenon of clonal dominance.

In addition to providing insights into the heterogeneity and expression of the murine B-cell repertoire, these studies point out fine structural relationships between PC-specific clonotypes. It is clear that the PC-specific B-cell repertoire of C3H, A/He, AKR, and BALB/c mice share variable region determinants to varying extents. The retention of some variable region determinants in strains differing in allotype may imply strong selective pressure for retention of certain specificities. This conclusion is best exemplified by the finding of the R T15 idiotypic determinant on BALB/c monoclonal antibodies with and without reactivity to M anti-T15. The identification of idiotypic determinants in some antibodies in conjunction with M T15 idiotypic determinants and independently on others has important implications for the genetic relationship between the two determinants. The occurrence of the T15 clonotype in high frequency in every individual of the BALB/c strain suggests that determinants detected by both anti-idiotypic preparations are encoded in the germ line. The presence of R T15+, M T15-antibody in BALB/c may indicate that this combining site or very similar sites can be associated with other framework sequences as well. If this binding site amino acid sequence is important for the survival of the organism, as suggested by its conservation within the species, it would be logical to assume that it may be associated with framework residues other than the one defined by M anti-T15. This expression may be the result of the fortuitous generation of similar variable region determinants either evolutionarily (gene duplication and segregation) or somatically (e.g., via episomal insertion [14]). Amino acid sequence analysis of the R T15+, M T15− monoclonal antibodies may be essential for discriminating between these possibilities.

Summary

The idiotypic identification of monoclonal antibodies has been used to define and enumerate clonotypes within the murine repertoire of B cells specific for phosphorylcholine (PC). The response in the BALB/c strain is dominated by a single antibody specificity which is identical to TEPC 15 protein; however, antibody without the TEPC 15 idiotype appears heterogeneous by idiotypic cross-reactivity and hapten inhibition of binding to antigen. Dissection of the PC-specific repertoire in the AKR, A/He, and C3H strains has indicated that some monoclonal antibodies share binding-site idiotypic determinants with TEPC 15, although these clones represent a minority of the precursor cells.
In addition to providing insights into the heterogeneity and expression of the murine B-cell repertoire, these studies emphasize structural relationships between PC-specific clonotypes. Within the BALB/c strain, some antibodies share combining-site-related idiotypic specificities with TEPC 15, but differ in other variable region determinants. Among allotypically distinct strains, there exists a remarkable similarity of variable region determinants in at least a minority of antibodies.

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