ANTI-PHOSPHOCHOLINE HYBRIDOMA ANTIBODIES

I. Direct Evidence for Three Distinct Families of Antibodies in the Murine Response*

BY J. LATHAM CLAFLIN, SUSAN HUDAK, AND ANNE MADDALENA

From the Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Three major groups of antibodies have been identified during the analysis of the clonal response to phosphocholine (PC). The predominant one shows multiple molecular features of the PC-binding myeloma protein (PC-BMP) TEPC 15 (1–3), and two others possess idiotypes (Id) and L-chain characteristics of two other PC-BMP, M511 (2, 4) and M603 (5). All three Id groups are conserved in inbred mice (1, 2, 4–6). Serological studies indicate that antibodies sharing Id with M511 or M603 are both heterogeneous, i.e., each is a distinct family of antibodies whose members appear to be closely related to each other (4, 5). In contrast, normally expressed T15 Id+ antibodies appear to be virtually homogeneous (6), although in one \( Igh \) haplotype we have recently identified at least three distinct antibodies that bear T15 Id (7).

To evaluate the clonal response at both the protein and chromosomal levels, we have generated and studied a large series of hybridoma cell lines from different strains, all produced by fusion with the nonsecreting cell line SP2/0-Ag14. The present investigation describes the relationships among these hybridomas, with particular emphasis on family membership, L-chain features, and antigen-binding characteristics. We find that hybridoma anti-PC antibodies are divided into three families, each of which resembles one of the three major groups of antibodies seen after immunization. The antigen-binding properties of the hybridoma anti-PC antibodies in a family are similar to each other and to the prototype PC-BMP, with one striking exception. Moreover, we have found that there is not necessarily a direct correlation between binding specificity and idiotypic or chain structure. Thus, we have verified our previous findings on the composition and conservation of the anti-PC repertoire in mice but suggest in addition that structural variation (including idiotypic) may not always translate into demonstrable changes in antigen-binding diversity.

Materials and Methods

Mouse Strains. BALB/c, AKR, and CBA mice were obtained from our mouse colony, which has been developed from breeding stock obtained from The Jackson Laboratory, Bar Harbor.

* Supported by grant AI-12533 from the National Institutes of Health and by grant IM-157 from the American Cancer Society.

1 Abbreviations used in this paper: AP, alkaline phosphatase; BMP, binding myeloma protein; BSA, bovine serum albumin; FCS, fetal calf serum; GPC, \( \alpha \)-glycerophosphocholine; HAT, hypoxanthine, aminopterin, and thymidine; HP, hybridoma proteins; Id, idiotypes; IEF, isoelectric focusing; PC, phosphocholine; PNOC, phosphonocholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRIA, solid-phase radioimmunoassay.
Maine. BABI4 mice were obtained from Dr. M. Weigert (Institute for Cancer Research, Philadelphia, Pa.). All other mice were obtained directly from The Jackson Laboratory. Mice were 10-14 wk of age when immunized.

Myeloma and Hybridoma Proteins (HP). The PC-BMP, T15, M603, M511, and M167 were purified from PC-Sepharose by affinity chromatography as previously described (2). Anti-PC HP were purified by the same procedure from either tissue culture fluid or ascites, depending on whether the cell line was propagated in vitro or in vivo. HP from primary clones were obtained from the first 5-10 ml of tissue-culture fluid accumulated during the initial phase of hybridoma propagation. Purity and monoclonality were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) in polyacrylamide gels (2). The L-chain type was determined by IEF of SDS-PAGE-purified L chains as previously described (2). This method permitted direct examination of antibodies from tissue culture fluid, where the total yield of antibody was often 50-100 μg.

Idiotypic and Isootypic Antisera. The preparation of rabbit antisera to T15 (C57 anti-T15) and M511 (C70 anti-M511) have been described previously (4, 8). Anti-idiotypic antiserum to M603 Id+ antibodies (Gp 17 anti-M603) was prepared in guinea pigs using anti-Proteus morganii (Potter) antibodies (5). These contain a preponderance of antibodies idiotypically and structurally related to M603. Purified anti-P. morganii antibody pooled from two C57BL/6 mice, 12-5 and 12-7, were used as the ligand in this assay. Each antiserum was adsorbed with normal mouse serum and appropriate myeloma proteins coupled to Sepharose 4B to remove anti-isotype and cross-reactive anti-idiotypic activity. Each of these anti-Id antisera recognized multiple members of the same but not other Id families. A fourth antiserum, C42 anti-VH-PC, has been described previously (9). This anti-idiotypic antiserum recognizes VH determinants common to all PC-binding myelomas, hybridomas, and serum antibodies. Each antiserum was specific, as demonstrated by direct and indirect (inhibition) solid-phase radioimmunoassays (SRIA) using a battery of 125I-labeled myeloma and hybridoma antibodies. The isotype-specific antisera to μ, γ1, γ2, and γ3 used in these experiments have been described in detail elsewhere (8). A polyvalent rabbit antiserum to mouse gamma chains was obtained from Gateway Immunosera (St. Louis, Mo.) and used after adsorption on a TEPC 183-Sepharose-4B column.

Idiotype and Isootype Assays. Quantitative SRIA for idiotypic and isotypic determinants were performed in polyvinyl microtiter plates as previously described (6). In some instances the IgG subclass was additionally examined with 125I-anti-isotype antibody as a probe of isoelectric-focused antibody. Whether hybridoma antibodies belonged to the IgM or the IgG class was also determined by SDS-PAGE of intact or reduced antibody, using specific marker proteins, including known IgM, IgG, and IgA myeloma and/or HP (10).

Production, Screening, and Selection of Anti-PC HP

Cell Lines and Media. The myeloma cell line used for fusion was SP2/0-Ag14, a nonsecreting variant of X63 (MOPC 21) (11). The cell line was maintained in suspension in Dulbecco’s modified Eagle’s medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with glutamine, penicillin, streptomycin, nonessential amino acids, and 15% heat-inactivated fetal calf serum (FCS). Somatic cell hybrids were selected in Iscove’s modified Dulbecco’s medium (Grand Island Biological Co.), supplemented with 20% FCS and hypoxanthine, aminopterin, and thymidine (HAT). Primary clones were subcloned in soft agar, using thymocytes as feeder cells (12). Cell lines and hybridomas were grown at 37°C in a humid atmosphere of 5% (cell lines) or 7% (hybridomas) CO2 in air.

Generation of Anti-PC Hybridomas. Spleen cells used for fusion were obtained from mice injected intraperitoneally on day 0 with either 10⁶ Streptococcus pneumoniae (R36A) or 100 μg PC-keyhole limpet hemocyanin (KLH) in complete Freund’s adjuvant, and on days 7-14 with the same antigen in saline. 2-12 wk later, mice were given an intravenous boost of antigen in saline and used for fusion in 3 or 4 d. Single-cell suspensions were prepared and the erythrocytes were lysed with 0.17 M NH4Cl. Polystyrene glycol-mediated fusion was performed according to a modification of the basic protocol of Gefter et al. (10, 13). Anti-PC hybridomas were detected by two procedures, each employing enzyme-linked immunoassays (14). In both methods 50 μl of tissue-culture fluid from growing clones was added to wells of a 96-well polystyrene microtiter plate that had been precoated with PC-bovine serum albumin (PC-BSA). After 4-6 h, the
plates were washed and each well was filled with 200 µl of alkaline phosphatase (AP)-conjugated rabbit anti-µ, or AP-rabbit anti-IgG (polyvalent) (method 1), or with 200 µl of AP-rabbit anti-VH-PC (method 2). After a further 16-24 h, the plates were washed and 100 µl of p-nitrophenyl phosphate in 0.5 M carbonate buffer, pH 9.8, was added to each well. Visual screen permitted detection of positive clones. These clones were propagated in Iscove’s HAT, subcloned, and stored frozen. Selected hybridomas were grown either in Iscove’s HAT to mass culture or in ascites in pristane-primed H-2 compatible F₁ mice.

Antigen-binding patterns of anti-PC hybridoma antibodies. The binding pattern of each HP for the choline analogues PC, 1-α-glycerophosphocholine (GPC), phosphonocholine (PNOC), and choline (C) itself was assessed in a competitive SRIA. A comparison was made of the molar concentration of the C analogues required to inhibit the reaction between antibody-coated plates and 125I-PC-bovine gammaglobulin. The ratio of efficiencies of binding at 15% determined for each hapten analogue in relation to PC binding (15). This provided a useful and reproducible assay for accurately measuring and comparing the fine-specificity binding patterns of different anti-PC antibodies.

Results
Among the more than 400 anti-PC hybridomas observed after fusion, we found considerable variation in their frequency/fusion, Ig class, and subclass, but not in idiotype (Table I). A similar observation with a limited number of fusions, using the secreting cell line 45.6TG.1 (MPC11), was made previously (10) and these data are incorporated into Table I. The predominant isotype was IgM, regardless of immunogen or time selected for fusion after a boost. IgA anti-PC hybridoma antibodies appeared frequently after fusion of R36A-immune spleen cells with 45.6TG.1 but were not seen when PC-KLH was used as the immunogen (10). Idiotyping of HP consistently yielded three major groups, regardless of strain. These shared idiotypic determinants with T15, M511, or M603. 0-4% of hybridomas obtained from an R36A-immune or a PC-KLH-immune mouse (where VH-PC Id + hybrids were screened) did not type for one of these three idiotypes, although in some instances not all hybrids were screened with each antiserum. Furthermore, no HP has yet been found to be doubly positive for Id. For example, fusion number 55, using R36A-

| Table I
| Screen of Primary Clones |
|--------------------------|
| Mouse strain | Fusing cell line | Number of fusions | PC+/total | Ig class* | Idiotype‡ |
|---------------|------------------|-------------------|-----------|-----------|-----------|
| BALB/c        | 45.6TG.1         | 4                 | 25/52     | IgM, IgA  | T15, M511 |
|               | SP2/0            | 8                 | 25/610    | IgM, IgG, | T15, M511, |
|               |                  |                   |           | IgG3      | M603      |
| C58           | SP2/0            | 2                 | 7/82      | IgM       | T15, M511, |
| CBA           | SP2/0            | 2                 | 108/475   | IgM, IgG2 | T15, M511, |
|               |                  |                   |           |           | M603      |
| BAB14         | SP2/0            | 2                 | 17/59     | IgM, IgG3 | T15, ?    |
| PL            | SP2/0            | 1                 | 102/149   | IgM, IgG2 | T15, M511 |
| AKR           | 45.6TG.1         | 2                 | 27/63     | IgM, IgA  | T15, M511 |
|               | SP2/0            | 2                 | 27/148    | IgM, IgG  | T15, ?    |

* Determined by enzyme-linked immunosorbent assay and SDS-PAGE (IgM or IgG), SRIA (all classes and subclasses), IEF (IgG subclasses). In some cases the IgG antibodies were not typed for subclass.
‡ Determined by SRIA with idiotype-specific antisera to T15, M511, and M603. ? indicates that not all hybridomas were tested with each anti-idiotypic antiserum.
immune BALB/c spleen cells, contained 45 T15 Id⁺, 7 M511 Id⁺, 18 M603 Id⁺, and 2 HP, which were V₁h-PC Id⁺, but negative for the major group-specific Id. A second fusion involving CBA spleen cells, number 101, contained 58 T15 Id⁺, 11 M511 Id⁺, 6 M603 Id⁺, and 1 untyped HP.

Idiotypic Analysis. To determine possible relationships among individual HP, idiotypic analyses of a number of antibodies were performed. These HP were selected only on the basis of the initial qualitative screen and the growth stability of the hybridoma line in culture. The antisera used were directed against T15, M511 PC-BMP, or an M603 Id⁺ antibody from A/He mice. Each was capable of detecting a distinct family of closely related antibodies expressed in the serum of R36A- or PC-KLH-immune mice, regardless of strain (4-7).

Table II shows the results of the idiotypic analyses. The HP from the cloned cell lines and their sublines fell into the same three Id clusters seen in the screen of primary clones. We designated these the T15, the M511, and the M603 HP families, because Id and IEF data on serum antibodies have shown heterogeneity among Id⁺ molecules (4-7). Subcloning showed that two of the primary clones, 101.6E5 and 101.6G2, actually consisted of two idiotypically different clones. Among T15 Id⁺ HP, a wide range of I₅₀ was observed in patterns that seemed to reflect class as well as Igh haplotype differences. IgM antibodies from BALB/c (Igh⁺) and CBA (Igh⁺) gave I₅₀ values between 75 and 230 ng/ml. This is within the range of the error of the assay, and therefore the values are not statistically different from each other. Three IgM HP from AKR (Igh⁺), C58 (Igh⁺), and PL (Igh⁺) gave I₅₀ values 5-10 times those obtained with BALB/c HP. Interestingly, all three possessed an Igh-Pc-A L chain, whereas BALB/c and CBA have an Igh-Pc-B L chain (16). BAB14 HP 59.6C5 and other IgG3 HP (data not shown) regularly gave excellent inhibition, but it is not known whether this is due to the subclass, to Igk haplotype, or to the fact that the strains of origin belong to a group of Igh⁺ mice that exhibit multiple T15 antibodies (7). More detailed analysis of additional hybridomas of different IgG subclasses and their associated idiotypes will be presented elsewhere.

All the M511 Id⁺ HP tested came from CBA mice and expressed the family idotype to approximately the same degree. As was found for T15 HP, IgM antibodies in the M511 HP family were poorer inhibitors than the IgA prototype antibody.

The M603 Id⁺ HP observed in BALB/c and CBA mice all expressed the common idotype found in this family and gave similar inhibitory patterns in the assay.

Although the results presented above show that anti-PC HP from different strains are directly related idiotypically to serum anti-PC antibodies, they do not show whether the HP within a family are idiotypically homogeneous or heterogeneous. Each anti-Id antiserum detects what appears to be multiple clonal products in IEF studies of serum antibodies.

L-Chain IEF Analysis. To further assess relationships among HP, isolated reduced and alkylated L chains from HP were analyzed by IEF. This procedure has routinely permitted typing of L chains among anti-PC antibodies, PC-BMP, and HP (2, 10, 15). The L chains of all HP listed in Table II, 10 additional T15 Id⁺, and two additional M603 Id⁺ antibodies from among the same fusions were examined. Typical results are shown in Fig. 1. All 26 T15 Id⁺ HP displayed an L chain that cofocused with T15 L chains (e.g., 55.2D3, 55.1G3.1, and 59.6C5.1). HP originating from AKR, PL, and C58 (e.g., 103.1C9.1), (10), showed the electrophoretic shift characteristic of
Table II

### Idiotype Analysis of Hybridoma Anti-PC Antibodies

| Hybridoma antibody | Anti-PC family | Strain | Ig class | Igζ‡ |
|--------------------|----------------|--------|----------|------|
| 15.1               | T15            | BALB/c | IgM      | 185  |
| 15.2               | T15            | BALB/c | IgM      | 218  |
| 15.3               | T15            | BALB/c | IgM      | 127  |
| 15.4               | T15            | BALB/c | IgM      | 75   |
| 15.5               | T15            | BALB/c | IgM      | 89   |
| 55.1G3             | T15            | BALB/c | IgM      | 95   |
| 55.1G3.1§          | T15            | BALB/c | IgM      | 120  |
| 55.2D9§            | T15            | BALB/c | IgM      | 230  |
| 101.3G2            | T15            | CBA    | IgM      | 165  |
| 101.3G9§           | T15            | CBA    | IgM      | 182  |
| 101.3D3            | T15            | CBA    | IgM      | 110  |
| 22.1A4.3           | T15            | AKR    | IgM      | 950  |
| 59.1G3.2§          | T15            | C57    | IgM      | 500  |
| 103.3C3.2§         | T15            | PL     | IgM      | 2,500|
| 100.6G2.1          | T15            | CBA    | IgG2     | 1,000|
| 103.1C9.1§         | T15            | PL     | IgG2     | 600  |
| T15§               | T15            | BALB/c | IgA      | 42   |
| 101.3C2            | M511           | CBA    | IgM      | 48   |
| 101.3C2.2§         | M511           | CBA    | IgM      | 40   |
| 101.3G8.4§         | M511           | CBA    | IgM      | 36   |
| 101.6E5.4§         | M511           | CBA    | IgM      | 35   |
| 101.6G6            | M511           | CBA    | IgM      | 42   |
| 101.6G6.2§         | M511           | CBA    | IgM      | 33   |
| M511§              | M511           | BALB/c | IgA      | 18   |
| 55.6F3.4§          | M603           | BALB/c | IgM      | 521  |
| 55.7C8.4§          | M603           | BALB/c | IgM      | 461  |
| 100.6F9.1§         | M603           | CBA    | IgM      | 501  |
| 100.6G2.2§         | M603           | CBA    | IgM      | 291  |
| M603§              | M603           | BALB/c | IgA      | 861  |

* Laboratory designation of hybridoma antibody m.n.o, where m refers to fusion number, n refers to clone number, and o to subclone number. All hybridoma antibodies contained a single molecular species.

‡ Concentration of HP antibody giving 50% inhibition in idiotype assay; C57 anti-T15 Id vs. 125I-T15 for the T15 family and C70 anti-M511 Id vs. 125I-M511 for the M511 family.

§ HP tested for binding specificity in Fig. 3.

|| Percent inhibition of the reaction Gp 17 anti-M603 IdX vs. 125I-Ab 12-5 when tested at 1 μg/ml.

T15 Id* anti-PC antibodies of the Igk-Pc-A phenotype (16). Similarly, the L chains of M511 Id* HP (e.g., 101.6G6.2, 101.6E5.4, and 101.3C2.2) cofocused with the L chains of M511, but with one difference. All showed an in-phase shift by one position toward a higher isoelectric point (pI). Five of the six M603 Id* HP contained L chains that cofocused with M603 L chains (e.g., 100.6F9). The sixth (100.6G2.2) showed an in-phase shift similar to that seen for M511 Id* HP, but in this case it was toward a lower pI. Similar shifts in pI could also be observed in the H chains of some HP (data not shown). In the one observed instance, two IgG2b T15 Id* HP from CBA contained...
T15 L chains that cofocused with each other; however, the H chains focused in phase but out of step by one band. The intact HP focused in phase but out of step by two bands. This is consistent with a net single-charge difference per H chain and two per molecule. These results show a striking positive correlation between Id and L chain type, confirming an earlier association observed with serum antibodies (2). Moreover, L and H chains within an HP family appear to be quite similar to each other, although there are clear instances where they are not identical.

**Binding-Site Specificity.** A measure of the binding site of the anti-PC HP was obtained by analyzing the reactivity of the HP for various choline analogues (GPC, PNOC, and C) relative to that of PC (9, 15). By this criterion the PC-BMP T15, M511, and M603 display distinct binding-site activity. In the initial experiments we tested HP purified from the tissue culture of growing primary clones of 26 T15 Id+, 5 M603 Id+, and 6 M511 Id+ hybridomas (Fig. 2). In the second series of experiments, we tested representative HP purified from subclones grown as ascites; 12 T15 Id+, all 5 M603 Id+, and the original 5, and an additional M511 Id+ HP were examined (Fig. 3). A summary of the results is provided in Table III. The analysis revealed three major reactivity patterns among the HP. Group A (×), contained HP from nine fusions and six different strains, which were T15 Id+ and IEF-T15 L+. The binding profile of each corresponded closely in fine specificity to that of T15. A second group B (○), derived from BALB/c and CBA was indistinguishable from M603 in its binding profile. Group C contained one group of five (○) which behaved similarly, and a sixth, 100.3C2 (●), which displayed a unique pattern. Neither group possessed a profile resembling that of M511 or of M167 (whose pattern is similar to that of M511, data not shown). T15 and M603 HP were distinguished from each other by their reactivity with PNOC. M511 HP could be differentiated from T15 HP by the
same criterion and from M603 HP in their reactivity with C. Some differences occurred in the assay of C binding in the primary clones and their subclones, but this is probably explained by contaminating FCS proteins in HP purified from tissue-culture fluids. The PC-BMP used in both sets of assays were obtained from ascites fluid. Thus, T15 and M603 HP, regardless of strain, display a striking similarity to the BALB/c PC-BMP, T15 and M603, respectively. By contrast, the M511 HP, which were derived from CBA, displayed two different types of binding-site activity and both were distinctly unlike that of M511.

Discussion

The present investigation describes immunochemical characteristics of more than 30 anti-PC HP, from among over 400, which were derived from hybridomas that secreted only two known Ig polypeptide chains, the H and L chains of the spleen cell partner. HP from seven genetically distinct strains were included in this study. All HP tested possessed specificity for PC and contained an H-chain idiotypic determinant VH-PC, which is shared by anti-PC antibodies arising in immunized mice. Among the HP, three well-defined groups or families (vide infra), which we have designated the T15, the M511, and the M603, were observed. A fourth and minor group that appeared to be negative for the three major idiotypes, although all of its members were VH-PC Id⁺, was also observed. Each of the three major groups possessed several biochemical and immunochemical markers that were characteristic for each of its members. In the T15 HP family there was a remarkable degree of conservation in the binding activity and L-chain IEF pattern that occurred regardless of strain, class, or
Fig. 3. Binding-specificity profiles for anti-PC HP purified from ascitic fluid. Symbols are the same as those in Fig. 2. Cell lines and their subclones tested are denoted in Table II by §. Additional HP included were: T15 HP, 1 BALB/c, 2 CBA, and 2 PL; M511 HP, 2 CBA; M603 HP, 1 CBA.

Table III

Summary of Antigen-binding Specificity Patterns in Hybridoma Antibodies

| Strain  | Genotype | Number of fusions | Hybridoma antibodies tested | Idiotype family* | Specificity pattern‡ |
|---------|----------|-------------------|----------------------------|------------------|----------------------|
| BALB/c  | a b      | 3                 | 14                         | T15              | T15 14               |
| C58     | a a      | 1                 | 1                          | T15              | 1                   |
| CBA     | j b      | 2                 | 6                          | T15              | 6                   |
| BAB14   | a/b b    | 1                 | 2                          | T15              | 2                   |
| Pl      | j a      | 1                 | 9                          | T15              | 9                   |
| AKR     | d a      | 2                 | 8                          | T15              | 8                   |
| BALB/c  | a b      | 1                 | 3                          | M511             | 1 (2)§               |
| CBA     | j b      | 1                 | 6                          | M511             | 6                   |
| AKR     | d a      | 1                 | 1                          | M511             | (1)§                 |
| BALB/c  | a b      | 2                 | 3                          | M603             | 2                   |
| CBA     | j b      | 1                 | 2                          | M603             | 2                   |

* Assessed by SRIA with idiotype-specific antisera and L-chain IEF typing.
‡ The reactivity pattern of the antibodies for C analogues was determined as described in Materials and Methods and compared to the PC-BMP, T15, M511, and M603.
§ These HP were derived from fusions with 45.6TG.1 and all contained MPC11 L chains in addition to the PC-specific H and L chains (10).

subclass. M603 HP followed a similar pattern. The M511 HP appear to be an exception, because considerable differences in binding specificity were observed among the antibodies. This group was derived from one strain, CBA, but its members still
contain L chains that focus similar to M511 L chains and that bear an M511 idiotypic determinant.

The Id that help define a family are interesting. Each HP so far examined possesses one of the family-specific Id and not another. It is a marker for that family and not another; e.g., absorption of a heterologous anti-T15 with a member of a different family restricts the antisera. It is as if the Id identified the same molecular region and were therefore mutually exclusive. For example, they may be V₅ specific. Alternatively, there may be molecular constraints that permit only certain types of V-region combinations in anti-PC antibodies. Sequence analysis of selected hybridomas now in progress should help resolve this point, but it is clear that the anti-Id with which we have worked identify nonoverlapping populations of anti-PC antibodies in serum and among HP.

What is an HP family? Among anti-PC HP, a family is defined as a collection of molecules sharing idiotypic determinants, L-chain IEF profiles, fine specificity of binding, or combinations of these traits, but demonstrating differences in one of these parameters. For example, M511 and the M511 HP were quantitatively similar in an M511 Id and their L-chain L chain, but differed in their binding activity for choline analogues. HP families defined by Id, and in some cases by structure, have been identified in other immune response systems, such as the dextran (17), the arsonate (18), and the GAT (poly-[L-glutamic acid, L-alanine, L-tyrosine]) (19, 20). The classic example can be found in the original paper on cross-reactive idiotypy published in 1968 by Kunkel and his colleagues (21). In this paper and its extension (22), the investigators recognized multiple idiotypic subgroups of human IgM cold-agglutinins, most of which were also shown to contain individually specific determinations. The subgroups correlated well with specificity for blood group antigens. Related observations were made by Capra (23, 24) among human myeloma proteins showing anti-γ-globulin activity. A major extension of these works is reported here in the PC system in mice. We have apparently identified all or at least the major responding families of antibodies in one particular response. This corroborates earlier studies of three major idiotypes and L-chain types in the serum of PC-immunized mice (2, 6). Moreover, we have demonstrated that each HP family can be defined equally well by fine specificity of binding, L-chain type, or both, in addition to the trait of idiotypic.

A family of anti-PC HP is also normally heterogeneous, even within a strain. This heterogeneity was revealed by differences in any of the three parameters. The relationships among these variables are intriguing, because it appears that the heterogeneity observed within a family operates within certain guidelines. T15 HP, the largest group obtained, are strikingly similar in their L-chain IEF pattern and their binding activity for choline analogues. There is no evidence to date that isotype, strain variation at the IgK or IgH locus, or degree of expression of idiotypic affect the binding specificity. In studies to be reported elsewhere we have extended the number of hapten analogues used to 20 and still have found no differences among the HP 103.1C9.1, 59.6C5, and the PC-BMP T15 (A. Maddalena, N. M. Young, and J. L. Claflin, unpublished observations). The T15 HP did show differences in the quantitative expression of T15 Id. For example, the CBA, AKR, C58, and PL HP were poorer inhibitors in the T15-anti-T15 assay than were BALB/c HP. The one BAB14 HP 59.6C5 was by far the best inhibitor. The antisera used to detect this Id,
as well as the other anti-idiotypic antisera, were not absorbed to make them HP or MP specific; they are family specific. They are undoubtedly heterogeneous in affinity and in specificity. Moreover, isotype and strain may influence this reaction. For instance, IgM HP from Igk-Pc\textsuperscript{a} mice were poorer inhibitors than those from Igk-Pc\textsuperscript{b} strains. The IgG3 HP was an excellent inhibitor, whereas the IgG2 HP were poor. Such isotype variation in Id expression was not observed among anti-Ars HP bearing the idiotype CRI (18). Perhaps we have identified heterogeneity within a strain (3) or between strains (7). Additional anti-PC HP of a single isotype and HP-specific anti-Id are required to evaluate the basis for heterogeneity among T15 HP, both within and between strains.

M603 HP are derived from only two strains, but they are genetically different at the \textit{Igh} locus. More important, they differ in \textit{V}_\text{H} at two (25) and possibly five (26) different residues. The potential for \textit{V}-region differences, even within a strain, seems great because anti-PC M603 Id\textsuperscript{*} serum antibodies appear to be heterogeneous (5). The HP give some evidence of Id heterogeneity and one of the two HP from CBA has an interesting in-phase shift in its L-chain IEF profile. This sort of pattern was also observed in M511 and T15 HP and has been observed among serum anti-PC antibodies with the T15 Id in CBA mice (7). We have suggested that the polypeptides affected are essentially identical and could be different members of the same \textit{V}-gene family (7). Despite these structural differences, the HP showed the same specificity for four choline analogues. Thus, there is no evidence that the structural diversity observed translates into demonstrable binding-site diversity. More extensive analysis of the binding sites of these HP and sequence analysis of them may prove valuable in determining structure-function relationships inasmuch as the data can be applied directly to the three-dimensional model of the Fab of M603.

The third group of HP, all of whose members share structural features related to the PC-BMP M511, is especially interesting. Its members all express the common M511 Id to the same degree. Their L chains focus with the L chains of M511, but as seen with the M603 HP, 100.6G2, all exhibit an in-phase shift with respect to M511 L chain by what appears to be a net single charge. This may be because all are of CBA origin, but serum anti-PC M511-like L chains from this strain cocoform with those of M511 itself (2). Thus, the third group is probably a subpopulation of M511 Id\textsuperscript{*} antibodies. In spite of these similarities, the HP contain binding profiles for choline analogues quite unlike the M511 profile. This may be due to the origin of the HP but it may also reflect the heterogeneity of M511 Id\textsuperscript{*} serum anti-PC antibodies (4). Further examination of these HP and others from \textit{Igh}\textsuperscript{*} mice should prove informative, but it is already clear that in this instance, idiotypy and L-chain IEF characteristics do not indicate a unique binding specificity.

At the present time we cannot state whether any two HP are identical. Clearly, this is probable because clonal expansion occurred before fusion and because hybrid cells were grown for 48 h before seeding in microwells. However, because the HP were derived from different strains, it is apparent that there was considerable conservation of Ig structure during evolution. Structural differences are known to exist in the anti-PC antibodies (24), but this has not resulted in a detectable change in the antigen binding specificity of T15 and M603 HP. The implications of this are not known, but analysis of these and additional HP with a greater array of choline analogues may reveal subtle differences in the binding site. It should be noted, however, that two
galactan BMP, which differ at nine positions in the hypervariable regions (27), possess the same specificity for 30 different carbohydrate haptons (28).

It is also not possible to state that we have obtained representative examples of most members of a serum anti-PC antibody family, although we seem to have made a start in the M511 family and do have an example of an IgG3 HP from BAB14 that cofocuses with the IgG3 T15 Id+ serum antibody from this strain (A. Maddalena and J. L. Clafin, unpublished observations). It should prove particularly exciting to relate HP to the diversity seen in serum Id+ antibodies and to analyze the structural and binding site diversity of the hybridoma population. It would then be possible to determine the genetic basis for the relatedness within an expressed family and whether Vh or VL is responsible, and, most important, how this correlates with the recent observations that V genes exist as series of small families of structurally similar gene segments (29).

Finally, we have observed a discordance in isotype and idiotype between serum anti-PC antibodies and anti-PC HP from the same immunization schedule. IgG3 and IgG1 antibodies are regularly observed in the anti-PC response to R36A and PC-KLH (6). However, IgG1 HP have not been observed, whereas IgG2 have been. We have no explanation for this but it has been observed by others (18, 30). Also of interest is the finding of a relatively high percentage of M511 and M603 Id+ HP in BALB/c, a strain that normally shows a virtual dominance of T15 Id+ antibodies after immunization with R36A or PC-KLH. HP structurally or serologically related to M603 and M511 have been observed in T15-suppressed but not in normal BALB/c (31). However, the small sample size studied may have precluded detection of M511- and M603-related HP in normal BALB/c. Our finding is reminiscent of the finding of a high percentage of anti-pneumococcal capsular polysaccharide HP in the normally nonresponder CBA/N 6 mouse (32) and supports the idea (5, 33) that B cells expressing M511 and M603 Id are actually present in BALB/c but fail to mature during the response.

Summary

Biochemical and serological studies were performed on more than 400 anti-phosphocholine (PC) hybridoma proteins (HP) derived from six strains of mice; 26 of these HP were examined in detail. All HP possessed specificity for PC, and all those tested contained an H-chain idiotypic determinant, Vh-PC, which is shared by PC-binding myeloma proteins (BMP) and anti-PC antibodies. Among the HP, three well-defined and distinct families that correlated well with previous studies on serum anti-PC antibodies were identified. The largest group shared idiotypic determinants, an L-chain isoelectric focusing (IEF) pattern, and a binding site specificity with the PC-BMP, T15. Using the same criteria, a second group was found to be strikingly similar to another PC-BMP, M603. The third group possessed an idiotypic determinant and an L-chain IEF profile similar to M511, but differences in binding site specificities were observed among the HP. The latter two groups contained members whose L-chain IEF profiles were not identical to other members of that group. Thus, among strains there is a remarkable degree of conservation among responding anti-PC antibodies, in both the kinds of anti-PC families that exist and the immunochemical and structural characteristics of various members within a family. Differences in at least one parameter were observed in each family, demonstrating that even a relatively
restricted response is heterogeneous. However, this diversity seems to operate within certain constraints.

We gratefully acknowledge the assistance of Virginia Davis and Cheryl Rasch, and thank Pat Dupke for her skilled help in the preparations of this manuscript.

Received for publication 30 September 1980.

References

1. Claflin, J. L., and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine IV. Idiotype uniformity of binding site-associated antigenic determinants among mouse antiphosphorylcholine antibodies. *J. Exp. Med.* 140:673.
2. Claflin, J. L. 1976. Uniformity in the clonal repertoire for the immune response to phosphorylcholine in mice. *Eur. J. Immunol.* 6:669.
3. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1977. The monoclonal antiphosphorylcholine antibody response in several murine strains: genetic implications of a diverse repertoire. *J. Exp. Med.* 145:876.
4. Wolfe, J., and J. L. Claflin. 1980. Clonal nature of the immune response to phosphocholine. IX. Heterogeneity among antibodies bearing M511 idiotypic determinants. *J. Immunol.* 125:2397.
5. Williams, K., and J. L. Claflin. 1980. Clonotypes of anti-phosphocholine antibodies induced with *Proteus morganii* (Potter). *J. Immunol.* 125:2429.
6. Claflin, J. L., and M. Cubberley. 1980. Clonal nature of the immune response to phosphocholine. VII. Evidence throughout inbred mice for molecular similarities among antibodies bearing the T15 idiotypes. *J. Immunol.* 125:551.
7. Claflin, J. L. 1980. Clonal nature of the immune response to phosphocholine. VIII. Evidence that antibodies bearing T15 idiotypic determinants in *Igh*1 mice comprise a family of antibodies. *J. Immunol.* 125:559.
8. Claflin, J. L., and M. Cubberley. 1978. Clonal nature of the immune response to phosphocholine. VI. Molecular uniformity of a single idiotype among BALB/c mice. *J. Immunol.* 121:1410.
9. Claflin, J. L., and J. M. Davie. 1975. Clonal nature of the immune response to phosphorylcholine. V. Cross-idiotypic specificity among heavy chains of murine anti-PC antibodies and PC-binding myeloma proteins. *J. Exp. Med.* 141:1073.
10. Claflin, J. L., and S. L. Wei. 1979. Control of V*κ* expression in the mouse. I. Unexpected expression of the V*κ* allele, *Igk-Pc* *, in a somatic cell hybrid of AKR (Igk-Pc*) origin. *J. Immunol.* 123:1051.
11. Shulman, M., C. D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature (Lond.*) 276:269.
12. Lernhardt, W., J. Anderson, A. Coutinho, and F. Melchers. 1978. Cloning of murine transformed cell lines in suspension culture with efficiencies near 100%. *Exp. Cell Res.* 111:309.
13. Gefter, M. L., D. H. Marguiles, and M. D. Scharff. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genet.* 3:231.
14. Keren, D. F. 1979. Enzyme-linked immunoabsorbent assay for immunoglobulin G and immunoglobulin A antibodies to *Shigella flexneri* antigens. *Infect. Immunol.* 24:441.
15. Claflin, J. L. 1980. Analysis of antibodies in the repertoire to pneumococcal phosphocholine by the use of mouse hybridomas. In Microbiology D. Schlessinger, editor. American Society for Microbiology, Wash. D. C. 186.
16. Claflin, J. L. 1976. Genetic marker in the variable region of kappa chains of mouse antiphosphorylcholine antibodies. *Eur. J. Immunol.* 6:666.
17. Clevinger, B., J. Schilling, L. Hood, and J. M. Davie. 1980. Structural correlates of cross-reactive and individual idiotypic determinants on murine antibodies to α-(1 → 3) dextran. J. Exp. Med. 151:1059.

18. Lamoyi, E., P. Estess, J. D. Capra, and A. Nisonoff. 1980. Heterogeneity of an intrastrain cross-reactive idiotype associated with anti-p-phenylarsonate antibodies in A/J mice. J. Immunol. 124:2834.

19. Pierres, M., S.-T. Ju, C. Waltenbaugh, M. E. Dorf, B. Benacerraf, and R. N. Germain. 1979. Fine specificity of antibodies to poly (Glu6°Ala3°Tyr a°) produced by hybrid cell lines. Proc. Natl. Acad. Sci. U. S. A. 76:2425.

20. Ju, S.-T., M. Pierres, R. N. Germain, B. Benacerraf, and M. Dorf. 1979. Idiotype analysis of anti-GAT antibodies. VI. Identification and strain distribution of the GA-1 idiotype. J. Immunol. 123:2505.

21. Williams, R. C., H. G. Kunkel, and J. D. Capra. 1968. Antigenic specificities related to the cold agglutinin activity of gamma M globulin. Science (Wash. D. C.). 161:379.

22. Feizi, T., H. G. Kunkel, and D. Roeleke. 1974. Cross-idiotypic specificity among cold agglutinins in relation to combining activity for blood group related antigens. Clin. Exp. Immunol. 18:283.

23. Kunkel, H. G., V. Agnello, F. G. Joslin, R. J. Winchester, and J. D. Capra. 1973. Cross-idiotypic specificity among monoclonal IgM proteins with anti-γ-globulin activity. J. Exp. Med. 137:331.

24. Capra, J. D., and J. M. Kehoe. 1974. Structure of antibodies with shared idiotypy: the complete sequence of the heavy chain variable regions of two immunoglobulin M anti-gamma globulins. Proc. Natl. Acad. Sci. U. S. A. 71:4032.

25. Claflin, J. L., and S. Rudikoff. 1979. Structural evidences for a polymorphic or allelic form of the heavy chain variable region. J. Immunol. 122:1402.

26. Rudikoff, S., and M. Potter. 1980. Allelic forms of the immunoglobulin heavy chain variable region. J. Immunol. 124:2089.

27. Rudikoff, S., D. N. Rao, C. P. J. Glaudemans, and M. Potter. 1980. K chain joining segments and structural diversity of antibody combining sites. Proc. Natl. Acad. Sci. U. S. A. 77:4270.

28. Jolley, M. E., S. Rudikoff, M. Potter, and C. P. J. Glaudemans. 1974. Structural requirements for the binding of derivatives of α-galactose to two homogeneous murine immunoglobulins. Biochemistry. 13:3179.

29. Seidman, J. G., A. Leder, M. H. Edgell, F. Polsky, S. M. Tilghman, D. C. Tiemeier, and P. Leder. 1978. Multiple related immunoglobulin variable region genes identified by cloning and sequence analysis. Proc. Natl. Acad. Sci. U. S. A. 75:3881.

30. Marshak-Rothstein, A., M. Siekevitz, M. N. Margolies, M. Mudgett-Hunter, and M. Gefer. 1980. Hybridoma proteins expressing the predominant idiotype of the antiazophenylarsonate response of A/J mice. Proc. Natl. Acad. Sci. U. S. A. 77:1120.

31. Kocher, H. P., C. Berek, M. H. Schrier, H. Cosenza, and J. Jaton. 1980. Phosphorylcholine-binding hybridoma proteins of normal and idiotypically suppressed BALB/c mice. II. Variable region N-terminal amino acid sequences. Eur. J. Immunol. 10:264.

32. Schroer, K. R., K. J. Kim, B. Prescott, and P. J. Baker. 1979. Generation of anti-type III pneumococcal polysaccharide hybridomas from mice with an X-linked B-lymphocyte defect. J. Exp. Med. 150:698.

33. Ruppert, V. J., K. Williams, and J. L. Claflin. 1980. Specific clonal regulation in the response to phosphocholine. I. Genetic analysis of the response of a distinct idiotype (M511 Id). J. Immunol. 124:1068.