A series of phosphorylcholine (PC) binding myeloma proteins of BALB/c origin have been described and characterized by several laboratories (1-4). One group of these proteins, including among others T15, H8, and S107, has been shown to possess identical variable region antigenic determinants (idiotypes) (2, 5) as well as identical partial variable region light and heavy chain amino acid sequences (6). This group of proteins also demonstrates the same binding specificity for PC and various analogs (3). Other PC binding proteins have been identified (M603, M167, M511, and W3207) which have different idiotypes (2, 4), variable region amino acid sequences (6-10), and specificity (3, 11). Studies in this laboratory and others (5, 11-14) have suggested that BALB/c mice immunized with PC-containing antigens respond by making antibodies predominantly of the T15 idiotype. We have further shown that rabbit antisera specific for an idiotypic determinant(s) in or associated with the combining site of the T15 group recognizes the same determinant in antibodies to PC elicited in BALB/c and 17 other mouse strains (5), suggesting a conservation in this species of at least one binding site for PC. The characterization of a PC-binding myeloma protein in CB 20 mice (BALB/c.B6 [IgCH]) with structural, functional, and idiotypic characteristics similar to T15 (15) further supports this contention.

In an attempt to ascertain the structural basis of the apparent binding site conservation we have begun to examine the primary amino acid sequence of antibodies to PC induced in various mouse strains. This system provides an opportunity to observe the evolution of the genes coding for these antibodies among inbred strains of the same species and to assess the genetic mechanisms compatible with the observed structures. In this regard we report here initial studies on the characterization of anti-PC antibodies in A/J mice.

Materials and Methods

Production and Purification of A/J Anti-PC Antibodies. A/J mice were obtained at 6 wk of age from The Jackson Laboratory, Bar Harbor, Maine. Beginning at 8 wk of age, and at biweekly intervals thereafter, 45 mice received six intraperitoneal injections of 10⁶ heat-killed (56°C, 30 min) Streptococcus pneumoniae str. R36A (11). Mice were bled 4 and 6 days after each injection. Sera obtained after the second injection all had high hemagglutinin titers and were pooled.
Antibodies with specificity for PC were isolated from a PC-Sepharose column by affinity chromatography (16). A total of 21 mg of antibody was obtained from 92 ml of serum; each mouse contributed on the average 460 μg of specific antibody from 2 ml of serum. Isolated anti-PC antibodies were IgM by immunoelectrophoresis and by immunodiffusion with class-specific antisera.

The BALB/c myeloma proteins T15, H8, W3207, M603, M511, and M167 were purified from ascitic fluid as previously described (16).

Isoelectric Focusing. A/J anti-PC antibodies and myeloma proteins were first completely reduced with 0.2 M 2-mercaptoethanol in 7 M guanidine in Tris-HCl buffer, pH 8.2 (2 h, 23°C) and alkylated with 0.4 M iodoacetamide ([tH]iodoacetamide, in some cases) (45 min, 4°C) (17). Heavy (H) and light (L) chains, separated on Sephadex G-100 in 1 M propionic acid-4.5 M urea, were analyzed by isoelectric focusing (IEF) in polyacrylamide gel slabs (15). After focusing gels were fixed in three changes of 5% TCA-5% sulfosalicylic acid and stained with Coomassie Brilliant Blue as described by Williamson (18).

Quantitation of Idiotypic Determinants. Rabbit antibodies with specificity for the binding site of T15, anti-T15's, have been described (19). Guinea pig antibodies having specificity for the binding site of M603, W3207, M511, and M167 (i.e., anti-M603s, -W3207s, -M511s, -M167s) were prepared in a similar fashion. Each idiotypic antibody was completely specific for the respective myeloma protein; in both direct and indirect radioimmunoassays (see below) no cross-reactions were observed. Moreover, the reaction between each anti-idiotypic antibody and its corresponding myeloma protein was greater than 90% inhibited by PC.

Quantitation of idiotype determinants in A/J anti-PC antibodies was examined by solid-phase radioimmunoassay (TBA) as previously described (5). The amount of an idiotype in the A/J anti-PC pool was determined by comparing its inhibition to that of a standard titration of myeloma protein for each of the five idiotype-anti-idiotypic assay systems. The sensitivities of the assays ranged from 0.01 to 0.05 μg/ml.

Sequence Analysis. 20 mg of A/J anti-PC antibody was reduced under conditions described above but in the absence of guanidine. After separation on a G-100 Sephadex column, H and L chain pools were dialyzed extensively against 1 M NaCl and distilled water and lyophilized.

Sequence determinations were performed on a Beckman 890C sequencer (Beckman Instruments, Inc., Palo Alto, Calif.) as previously described (8). Phenylthiohydantoin derivatives were identified by gas chromatography (GC) (20), thin-layer chromatography (TLC) (21), and HI hydrolysis to the free amino acids (22).

Results

Isoelectric-Focusing Patterns. A first assessment of the degree of heterogeneity in A/J anti-PC antibodies and their structural similarities to the PC-binding myeloma proteins was determined by isoelectric focusing of isolated L chains. Fig. 1 shows the stained L chain banding patterns of A/J anti-PC antibody and compares them to those of five PC-binding myeloma proteins. The L chains of M167 and H8 are not shown separately since they focus in positions identical to those of M511 and T15, respectively. T15 and H8 are structurally identical (6) through the first complementarity region, whereas M511 and M167 (7, 10) or M603 and W3207 (reference 6 and P. Barstad, personal communication) have structurally similar but not identical L chains. The L chain of each of the myeloma proteins appears in a set of closely grouped bands focusing in a narrow pH range of less than 1 unit which is indicative of a homogeneous polypeptide (23). The L chains of M603 and W3207 co-focus, but they differ in that the staining intensity of the bands is distributed differently. The L chain banding patterns of A/J anti-PC by comparison to the myeloma proteins are

2 Claflin, J. L. 1976. Uniformity in the clonal repertoire for the immune response to phosphorylcholine in mice. Eur. J. Immunol. In press.
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FIG. 1. (a) IEF patterns of L chains from A/J anti-PC antibodies and BALB/c anti-PC myeloma proteins. IEF was carried out in thin-layer polyacrylamide gel slabs in pH 5-9.5 ampholytes. (b) Interpretation of (a) showing that A/J anti-PC antibodies are composed of L chain indistinguishable from M511 (.), M603 (--), and T15 (••).

complex and range from pH 4.7 to 8.2. However, it is evident that bands corresponding in position to those of T15, M603 (W3207), and M511 (M167) are present. Because of their staining pattern, the middle set of bands in A/J appear more closely related to M603 than W3207. No additional bands were seen in this preparation even when the amount of sample focused was increased fivefold. Our interpretation of the results, shown in the right hand panel of Fig. 1, is that A/J anti-PC antibodies contain at least three immunoglobulins whose L chains are nearly identical to those of H8, M603, and M511 (M167). Quantitation of each L chain type by determining the radioactivity in each band after IEF of 14C-A/J anti-PC L chains gave 28% H8-like L, 38% M603-like L, and 33% M511-(M167-) like L.

Determination of Idiotypes. A portion of the pool of antibodies from A/J mice was diluted to 136 μg/ml and quantitated for idiotypic determinants characteristic of the available PC-binding myelomas. As shown in Table I each anti-idiotypic antibody was specific for its corresponding myeloma protein and was capable of accurately recognizing that protein in a pool of idiotypes. Analysis of the A/J anti-PC antibody pool revealed the presence of idiotypic determinants associated with the binding site of T15 and M511. Quantitative measurements of these two idiotypes indicated that each was present in about equal amounts and comprised approximately two-thirds of the antibody present in the pool. Binding site determinants of M167, M603, and W3207 were not detected in the pool, even though the assays were capable of measuring these idiotypes when present at a concentration of 10-50 ng/ml. Separate assays performed with additional guinea pig and rabbit antibody preparations to binding-site as well as nonbinding-site regions of W3207 (one antiserum), M603 (three antisera), and M167 (four antisera) failed to reveal the presence of immunoglobulin bearing W3207, M603, and M167 idiotypes (L. Claffin, unpublished data). Furthermore, attempts to detect immunoglobulins which shared cross-idiotypic determinants (24, 25) with M167
Table I

Concentration of Idiotypes in A/J Anti-PC Antibodies

| Antibody | Ig$ | Idiotypic determinant* |
|----------|-----|------------------------|
|          | µg/ml | % | µg/ml | % | µg/ml | % | µg/ml | % |
| A/J anti-PC | 136 | 41 | 30 | 52 | 38 | <0.01 | 0 | <0.05 | 0 | <0.05 | 0 |
| T15, M511, M603 | 33 each | 34 | 34 | 29 | 29 | <0.01 | 0 | 30 | 30 | <0.05 | 0 |
| T15, M167, W3207 | 33 each | 29 | 29 | <0.01 | 0 | 32 | 32 | 0.05 | 0 | 37 | 37 |

* Rabbit or guinea pig antibodies directed to the binding sites of T15 (anti-T15$^*_s$), M511 (anti-M511$^*_s$), M167 (anti-M167$^*_s$), M603 (anti-M603$^*_s$), and W3207 (anti-W3207$^*_s$) were used.

† Immunoglobulin (Ig) concentration of purified antibody.

§ Concentrations of anti-PC antibody (and percent of total Ig) giving 50% inhibition of binding of $^{125}$I-myeloma protein to the corresponding anti-idiotypic antibody was compared to a standard curve obtained with the appropriate myeloma protein. The error for each determination is ±15% of the calculated concentration. Mean results of three separate experiments are shown.

or M603 (anti-M167$^*_s$ or anti-M603$^*_s$) vs. $^{125}$I-A/J anti-PC antibody) were unsuccessful.

H Chain Sequence Determinations. Isolated H chains were subjected to 36 cycles of degradation. After analysis by GC and TLC the sequencer products from each degradation cycle were hydrolyzed to their free amino acids and applied to an amino acid analyzer so that the spectrum of all amino acids at any given position could be observed in the same assay. Quantitation of selected steps from the H chain determination are presented in Table II, and the sequence obtained is given in Table III. Significant heterogeneity was observed only at position 23 where Glx was found to be above background in addition to Ala.

L Chain Sequence Determination. L chains were analyzed as described above and the results are presented in Table IV. The L chain analysis was complicated by the fact that three different chains were being sequenced simultaneously as is evidenced by the results. At positions one, two, and three only a single amino acid was obtained (Asp, Ile, Val). At position four, two amino acids were identified (Met and Ile) in the ratio of approximately 2:1. At positions where two amino acids were identified in a 2:1 ratio the amino acid in higher concentration is listed twice for that position. After position 30 yields were no longer high enough to observe ratios as described above and the residues for positions 30 to 34 represent amino acids identified above background levels.

Discussion

Experiments such as those described above are essentially an attempt to analyze the gene products of a specific immune response at the structural level both qualitatively and quantitatively. In this regard it is appropriate at this point to comment on the limits of the sequence data presented. Since heterogeneity at any position in the sequence analysis may represent the occurrence of an additional gene, the methods and sensitivity of the techniques used for amino acid identification become limiting factors. It is important to point out that we are describing the results obtained from a pool of 45 mice rather than a single mouse. Occasional amino acid interchanges, which may or may not be significant genetic events, would be missed. Thus, the sequences presented in this
### Table II

**Phenylthiohydantoin Yields from Heavy Chain**

| Residue number | Amino acid | Total yield by GC (nM) |
|---------------|------------|------------------------|
| 2             | Val        | 25.9                   |
| 4             | Leu        | 28.4                   |
| 5             | Val        | 27.0                   |
| 8             | Gly        | 11.0                   |
| 11            | Leu        | 22.0                   |
| 18            | Leu        | 14.9                   |
| 23            | Ala        | 6.3                    |
| 27            | Phe        | 5.6                    |
| 32            | Phe        | 4.7                    |

### Table III

**H Chain Variable Region Sequences of Anti-Phosphorylcholine Antibodies**

- **A/J**
- **T15**
- **M603**
- **M167**
- **M511**

### Table IV

**L Chain Variable Region Sequences of Anti-Phosphorylcholine Antibodies**

- **A/J**
- **T15**
- **M603**
- **M167**
- **M511**

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* Sequences of T15, M603, and M167 are from Reference 6 and M511 from Reference 27.

† Ala was observed at this position by GC but not TLC and may be an artifact.

§ Amino acids in parentheses are tentatively identified.

**Positions 11 and 22 in M167 have previously been reported as Asp (D) and Thr (T) respectively (28). We have found these positions to be Asn (N) and Ser (S) (10).**
paper are those detected within these limitations and must be regarded as the major sequences in the response while not excluding the possibility of other structures at undetectable levels. For experiments involving induced antibodies, such as these, TLC is used in our laboratory only qualitatively to differentiate acidic and amide residues as the results are difficult to quantitate. All positions in these sequencer runs were initially analyzed by GC and results from the H chain are given in Table II. As may be seen at position 32 the total yield of Phe is 4.7 nM. 8% of the total sample was used for GC analysis or approximately 0.4 nM of Phe. This amount of material is close to the confidence limits of the assay. Thus, any other amino acids present at 50% or less of the major residue will likely be undetected. Heterogeneity in the early portion of the sequencer runs where yields are considerably higher would be detected at roughly the 10% level. Since only about one-half of the amino acid derivatives are adequately identified by GC the sequencer products at all positions were hydrolyzed to their free amino acids (22) and subjected to amino acid analysis. This method of identification while having about the same sensitivity as GC permits the observation of the levels of almost all amino acids in a single assay.

The present results provide three pieces of evidence which strongly imply that A/J and BALB/c mice have identical or nearly identical genes coding for antibodies to PC. Based on (a) L chain IEF patterns, (b) idiotypes, and (c) N-terminal structure of H and L chains, A/J anti-PC antibodies are strikingly similar to three BALB/c myeloma proteins that bind PC (T15, M511, M603). The results of the IEF experiments show that the L chains from antibodies produced to PC in A/J mice co-focus with L chains from BALB/c PC-binding myeloma proteins (T15 or H8, M511 or M167, M603 or W3207). Because this procedure measures principally charge differences one cannot assume that by this criterion alone each L chain type represents a single sequence (e.g. M511 and M167 have been reported to differ at a single position in their amino terminal 23 residues (7, 10, 26)) or that each L chain is associated with only a single H chain. It does, however, define limits in L chain heterogeneity that exist in the response and shows that the L chains produced in A/J anti-PC antibody are indistinguishable in charge composition from the L chains of PC-binding immunoglobulins of BALB/c origin. Idiotypic analysis, however, is highly discriminatory and capable of recognizing what must be small differences in structure. Indeed, in the present work there is a remarkable correlation between idiotype (as defined by these antisera) and structure. Idiotype analysis when correlated with the L chain percentage confirms the contention (5, 15) that the L chains in A/J anti-PC antibody, which have the same pI as T15 L, are derived from T15-like molecules. Furthermore, the distinction between M511- or M167-like antibody in the A/J anti-PC pool is readily made. Likewise A/J immunoglobulin(s) having an M603-like L chain is not totally like either M603 or W3207 even though its L chains have the same pI and are similar in structure to M603 L chains (see below). The failure to find the M603 idiotype in the A/J pool suggests that this L chain may be associated with an H chain other than that found in the BALB/c protein. However, in separate experiments we have found that every mouse strain tested to date produces some antibody with L chains that cofocus with M603 and that these M603-like antibodies from all strains share idiotypic determinants (J. L. Claflin, unpublished results).
Probably the most compelling data for the presence of identical or very similar genes in the two strains is the amino acid sequence analysis. When the sequence of the A/J H chain pool is compared to the four BALB/c H chains from PC-binding proteins (Table III) it can be seen that both the framework (positions 1–29) and first complementarity regions (positions 30–35) are essentially identical in all of the proteins with one exception. Val found at position four in M167 is not seen in the A/J pool. Position 23 in the A/J chains was found to contain both Ala and Glx while Ala is the only amino acid at this position in the BALB/c chains. Complete variable region sequences of the H chains from the BALB/c proteins have shown that T15, M603, M167 (8, 9), and M511 (27) have differences after the first complementarity region. Thus, it can not be determined at this time whether the A/J H chains are composed of a single molecular species or several as in the BALB/c proteins. Kluskens et al. (28), in an analysis of the H chains of BALB/c anti-PC antibody, found a single sequence for the first 20 residues, though, in this case, only the T15 idiotype was detected.

The L chain analysis while complicated by the simultaneous sequencing of three chains provides essentially the same results. By comparing the A/J sequences to the published BALB/c L chains (6, 7, 10) (Table IV) it can be seen that in A/J only a single amino acid was identified at positions one, two, and three (Asp, Ile, Val). The three BALB/c chains also all have Asp, Ile, and Val at the first three positions. Position four in the A/J sequence contained two amino acids, Met and Ile, in the ratio of 2:1. From the BALB/c sequences it can be seen that both T15 and M603 have Met at this position, whereas M511 or M167 has Ile. Thus the ratio for two Met:one Ile in the A/J sequence is consistent with the presence of three L chains identical to those of the BALB/c proteins. This type of analysis can be made for the first 30 positions of the L chain. At all positions where the three BALB/c chains have the same amino acid (1–3, 5, 6, 13, 19, 21, 22, 24, 26, 27), that amino acid alone was also found in the A/J sequence. At the other positions the amino acids present in the BALB/c sequences were likewise found in the A/J with the following exceptions. In the A/J chains Ala was detected at position 10 by GC but not TLC and, thus, may be an artifact. Position 18 appears to contain two Lys and one Ser residue, which suggests a substitution of Lys in the A/J chains for Arg found in M603. Position 21 contained Ile and Leu in addition to Met, which indicates an interchange to Leu for the Ile in either the T15 or M511 sequence. Arg at position 24 in the M167 chain was not identified but may be a technical problem as Arg in low yields is very difficult to analyze. Again, this analysis includes both the first framework portion of the L chain (positions 1–26) and part of the first complementarity region (from 27 on). The discrepancy between idiotyping and sequence analysis for M511- and M167-like antibody in A/J may be minor. M511 and M167 differ by a single amino acid in the first 23 residues which can be explained by a single base change. Thus, these two L chains may be extremely similar but associated with different H chains accounting for the idiotype differences.

The finding that essentially identical H and L chains are found in anti-PC antibodies from both A/J and BALB/c strains of mice implies that the genes coding for these structures are likewise very similar if not identical. This observation is striking in that A/J and BALB/c differ considerably in their
genetic makeup, and most importantly for these studies, at the H chain allotype locus. In terms of genetic mechanisms, it appears that for two unrelated strains to express the same primary sequences in an antibody response the genes coding for the respective H and L chains must be contained in the germ line. The likelihood of identical structures arising somatically by parallel mutation is extremely small. Several investigators have suggested the possibility that complementarity regions are coded for by an additional gene or episome which is inserted into a second gene coding for the framework portion of the variable region (29, 30). This mechanism also appears unlikely to be involved in the system described here for the following reasons. For the framework and the complementarity regions to be identical in the two strains but encoded by different genes, would require that identical parallel evolution of the two genes had occurred in each strain. Since the same complementarity regions appear to be associated with the same framework sequences in both strains, an insertional mechanism would further require that selection also take place at the framework level so that a specific framework sequence is always paired with the same complementarity region. Since the H chain sequence data extend only through the first complementarity region it is conceivable that the remainder of the H chains may be quite different from their BALB/c counterparts which would then make the data compatible with an insertional mechanism. The same point can be raised for the L chains. However, in this case the isoelectric focusing results further suggest that the L chains will be very similar to those in the BALB/c proteins. Furthermore, the idiotypic analysis again supports the contention that the uncharacterized portions of the variable regions (at least in the case of the T15 and M511-like proteins) will again be very similar to those of the BALB/c proteins. It seems to us the most plausible explanation of the data is that the framework and complementarity regions involved in the anti-PC immune response have coevolved and are coded for by a single gene. Selective pressure would then account for the maintenance of these genes in the two strains.

An important difference between these studies on induced antibodies and those from other laboratories is our finding of similarities not just within genetically identical individuals but between genetically unrelated members of a species. Capra et al. (31) and Freidenson et al. (32) have found that antibodies of defined idiotype from pooled serum or individual mice of a single strain (A/J) raised to an arsonate hapten are homogeneous in both their first H chain complementarity and framework regions. Cebra et al. (33) found that the sequences in the first 83 residues of the H chain variable region in three different antibodies to haptens from strain 13 guinea pigs were either unique or of restricted variability compared to normal immunoglobulin. It will be of great interest to see if the same sequences from anti-arsonate antibodies (31, 32) described in the mouse, or from anti-arsonate, anti-dinitrophenyl, and antitrimethylammonium antibodies (33) identified in the guinea pig can be found in different strains of mice or guinea pigs, respectively. In point of fact, groups of human myeloma proteins (which are obviously obtained from individuals in an outbred population) show remarkable similarities to the anti-PC system described above. A series of human cold agglutinins were found to fall into three major groups defined by shared idiotypic specificities (34). A second group of
human proteins with anti-gamma globulin activity has also been characterized (35). Two groups of these anti-gamma globulin proteins have been reported to share idiotypic specificities, although each protein has been shown to possess individual antigenic determinants. The complete H chain variable region amino acid sequence from two of the anti-gamma globulin proteins, Lay and Pom, has been determined by Capra and Kehoe (36). Only eight differences were found in the entire variable regions of which seven were from parts of the molecule which do not participate in antigen binding. The similarity of these proteins is remarkable considering their source and points out a high correlation between idiotyp (albeit crossreacting in this case) and primary structure. Thus, in view of our findings and those in humans one should anticipate considerable conservation of genetic information within a species. We are continuing to test this hypothesis by raising anti-PC antibodies in several other mouse strains as well as other species to see if the same set of genes can be identified and their products characterized.

Summary
Analysis of A/J antibody to phosphorylcholine (PC) revealed a striking degree of similarity to PC-binding myeloma proteins of BALB/c origin. By quantitative idiotypic analysis A/J anti-PC antibody was composed of antibodies bearing binding site idiotypic determinants indistinguishable from two different BALB/c myeloma proteins, T15 and M511. Idiotypic determinants of three other PC-binding proteins, W3207, M167, and M603 were not detected. Isoelectric focusing of the light chains verified the presence of antibodies similar to T15 and M511 and indicated the presence of a third antibody whose light chains had a pI identical to that of M603. When the sequence of A/J heavy chains was compared to the heavy chains of T15, M511, and M603, both the framework and first complementarity regions were identical in all cases. Sequences analysis of the light chains through part of the first complementarity region revealed three chains, one similar to each of the myeloma proteins T15, M603, and M167-M511. The latter two sequences differ by only a single amino acid (a single base substitution) in the first 23 residues, suggesting that the two light chains may be very similar if not identical.

Thus, BALB/c and A/J mice which differ genetically at multiple loci including the heavy chain allotype complex locus show a remarkable preservation of their anti-PC antibodies. These results indicate that the genes encoding these antibodies are contained in the germ line.

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