CLONAL NATURE OF THE IMMUNE RESPONSE TO PHOSPHORYLCHOLINE (PC)

V. Cross-Idiotypic Specificity Among Heavy Chains of Murine Anti-PC Antibodies and PC-Binding Myeloma Proteins*

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Williams et al. (1) in a study of monoclonal IgM cold agglutinins noted that antisera to different cold agglutinins possessed specificity not only for antigenic determinants unique to the individual proteins but also for determinants that were shared by cold agglutinins from a number of individuals. This phenomenon of shared or cross-idiotypy among immunoglobulins with similar specificity also has been demonstrated for human proteins with anti-γ-globulin activity (2) and among rabbit (3, 4) and mouse (5) antibodies to streptococcal group carbohydrates. In most instances the proteins exhibiting cross-idiotypic reactions had similar, but not necessarily identical, combining sites, and the idiotypic determinant shared by them was associated with the combining site (2, 5–8). Support for the structural similarity of antibodies with similar specificity and shared idiotypy has been provided by recent findings showing that IgM cold agglutinins, anti-γ-globulins (9, 11), or rabbit antibodies to streptococcal group C carbohydrate (7) have strikingly similar heavy and/or light chains.

Previous studies from our laboratory have demonstrated a remarkable similarity in the combining sites of mouse antibodies to phosphorylcholine (PC)[1] regardless of the genetic background of the mice. The antibodies from the mouse strains tested possessed indistinguishable binding specificities for a number of choline analogues (12) and had identical binding-site antigenic determinants (13). This suggests a striking conservation of the binding site region of mouse anti-PC antibodies, even though differences have been shown to exist in other portions of the variable region (14-16). In contrast, it was shown (17) that three PC-binding myeloma proteins that differed in combining specificity (18), as well as in idiotypes (19) and in light chains (17), have heavy chain sequences which are virtually identical through the first hypervariable region. These findings

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CGG, chicken gamma globulin; GPC, L-α-glycerophosphorylcholine; PC, phosphorylcholine.

imply that a prerequisite for all PC-combining activity in mice is a particular heavy chain subtype. The present study was carried out to test this hypothesis among PC-binding myeloma proteins and anti-PC antibodies isolated from a number of different mouse strains. Using a cross-idiotypic assay we found that each of six different PC-binding myeloma proteins and mouse anti-PC antibodies of different inbred strains possessed a common antigenic determinant which could be located primarily in the variable region of the heavy chain. Thus, V_H regions which are independent of the specific binding site have been conserved in mice.

Materials and Methods

Haptens and Hapten-protein Conjugates. Phosphorylcholine (PC), L-α-glycerophosphorylcholine (GPC), and choline (C) were obtained from Sigma Chemical Co., St. Louis, Mo. Calcium and cadmium ions in PC and GPC, respectively, were precipitated with phosphate before use. Chicken gamma globulin (CGG) and bovine serum albumin (BSA) were conjugated to PC as previously described (15).

Plasmacytomas. The plasmacytomas HOPC 8, TEPC 15, McPC 603, MOPC 511, MOPC 460, MOPC 384, MOPC 21 were obtained from Dr. M. Potter, National Cancer Institute, NIH, Bethesda, Md. The tumors were maintained by serial transfers in BALB/c mice.

Animals and Immunization. BALB/c, C58, C57BL/6, DBA/2, A, AKR, and CE mice (6–8 wk of age) were obtained from Jackson Laboratories, Bar Harbor, Maine. They were immunized intravenously on days 0 and 14 as previously described (15) with 10^8 pneumococci (R36A). Sera pooled from 10 mice per strain were collected 4, 6, and 8 days after the second injection.

Myeloma Proteins and Anti-PC Antibodies. Myeloma proteins and mouse IgM antibodies specific for PC were isolated by affinity chromatography from a PC-Sepharose immunoadsorbent (20) as previously described (13). CBPC 2, kindly provided by Doctors M. Potter and S. Rudikoff, NCI, is a myeloma induced in BALB/c · C57BL/ka (IgC_H) CB20 mice which produce a PC-binding IgA (κ) protein with the same binding specificity, amino-terminal heavy and light chain sequences, and binding site idiotypes as T15 and H8 (21). It carries, however, the heavy chain allotypic determinants of C57BL/ka (21). Another PC-binding myeloma protein, ALPC 43, provided by Dr. Potter originated in a congenic strain of BALB/c, BALB/c · AL/N IgC_H, which carries the IgC_H complex locus of AL/N mice (22). ALPC 43, which will be described in a later publication, belongs to the IgG3 class and carries neither the nonbinding site, T15 (16), nor the binding site, $H8_s$ (13), idiotypic determinant. M384 and M21 were purified by isoelectric focusing in an LKB 8100 liquid electrofocusing column using pH 5-8 Ampholines (LKB Instruments, Inc., Minneapolis, Minn.). M460, a dinitrophenyl-binding IgA protein, was a gift from Dr. R. Lynch of our department. The galactan-binding IgA (κ) myeloma proteins, J539 and X44, secreted by plasmacytomas J539 and xRPC 44 (23), respectively, were gifts from Dr. S. Rudikoff. Normal mouse globulin (NMG) containing IgM, IgA, and IgG was separated from pooled normal BALB/c serum by elution from DEAE-cellulose.

Affinity Labeling. Purified H8 was affinity-labeled with PC as described by Chesebro and Metzger (20). Monomer H8 at 10^{-5} M in 0.2 M borate, 0.15 M NaCl, pH 8.2, was reacted with *p*-diazonium phenylphosphorylcholine at a final exposure ratio of 4 moles reagent per mole of combining site. A paired experiment included 10^{-3} M PC as a site protector. As shown previously (24) the binding sites of site-labeled H8 were greater than 90% saturated with covalently bound PC, whereas site-unlabeled H8 reacted normally with PC.

Immunoglobulin Fractionation. Heavy and light chains of myeloma proteins and anti-PC antibodies were isolated after partial reduction and alkylation by chromatography on Sephadex G100 columns equilibrated with 1 M propionic acid, 4.5 M urea (25). The ascending and descending portions of the heavy and light chain peaks, respectively, were used. Based on radioactive markers, light chain contamination of heavy chains was less than 1%. Pepsin Fab fragments of M167 and T15 were prepared according to the procedure of Rudikoff et al. (26).

Preparation of Anti-idiotypic, Antiallotypic and Anti-kappa Antisera. An idiotypic antiserum to H8 protein was prepared in rabbit C1 as follows. 2 wk after the second monthly injection of 2 mg of HOPC 8 in complete Freund's adjuvant, serum was collected and passed over a Sepharose

immunoadsorbent coupled (27) with normal BALB/c serum containing 9 mg/ml of M460. As will be shown, this antiserum, Cl anti-H8, was idiotypically specific for H8 but possessed, in addition, antibodies specific for other myeloma proteins that bind PC. The preparation of antibodies specific for binding site-associated antigenic determinants of H8, anti-$H8_s$, has been previously described (24). Antiallotypic antiserum was prepared by immunization of A/J mice with M460 protein according to the procedure of Potter and Lieberman (22). This antiserum reacted only with IgA myeloma proteins and presumably detected the $A^{12, 13, 14}$ IgC_H determinants. A rabbit antiserum specific for mouse kappa chains was purchased from Gateway Immunosera Co., Cahokia, Ill. The gamma globulin fraction for use in the immunoassay described below was separated from each of these three antisera by DEAE-cellulose chromatography using 0.05 M phosphate buffer (PB), pH 8.0.

Detection of Idiotypic, Allotypic, and Isotypic Determinants. Distribution of idiotypic specificities among PC-binding proteins was examined by solid-phase radioimmunoassay (TBA) (28) using a modification of the procedure originally described by Kunkel et al. (2) for detecting cross-idiotypic specificities among monoclonal anti-γ-globulins. Briefly, microtest tubes (Beckman Instruments, Inc., Fullerton, Calif.) were coated with 0.2 ml of a limiting dilution of idiotypic antiserum to H8 as previously described (24). After rinsing the tubes with 0.15 M NaCl, 0.005 M PB, pH 7.2 (PBS), 0.21 ml of [^{125}I]M167 (1.9 ng/ml ~30,000 cpm/ml) in PBS containing 1% BSA, 0.1% normal rabbit serum and various concentrations of inhibitors was added. Tubes were incubated for 14–18 h at 37° C, rinsed with PBS, and the percentage inhibition of specific binding was determined. Alternatively, microtest tubes were coated with anti-kappa or with antiallotypic antisera to test for inhibition of binding to isotypic or allotypic determinants on [^{125}I]M167. In one instance a control experiment tested inhibition of binding to anti-$H8_s$ with [^{125}I]H8. Specific and nonspecific binding to antibody-coated tubes was 23%–29% and <0.5%, respectively, of added radioactivity.

Results

Specificity of Idiotypic Antiserum. The idiotypic antiserum to H8, when tested in the TBA using [^{125}I]H8 as the ligand, reacted strongly with H8, but only weakly with other myeloma proteins with related, but distinguishable, anti-PC activity (17-19) (e.g. M167, M603, and M511). Expressed quantitatively, a 1:100 dilution of the idiotypic antiserum maximally bound 30 ng of H8 and 3.6 ng of M167 under the conditions of the TBA. If, however, [^{125}I]M167 instead of H8 was used as the reference ligand in the assay, all PC-binding myeloma proteins were seen to inhibit binding to anti-H8-coated tubes equally well (Fig. 1 and Table I). This cross-idiotypic determinant was also found on CBPC 2, a

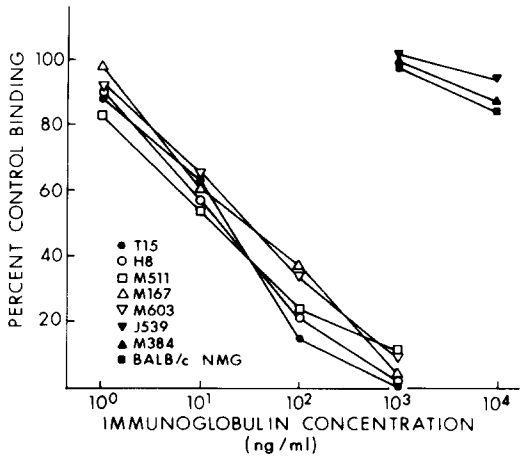

FIG. 1. Idiotypic similarity of PC-binding myeloma proteins. Purified myeloma proteins were tested for their ability to inhibit the binding of [^{125}I]M167 to Cl anti-H8.

TABLE I
Specificity of Idiotypic Antiserum

Myeloma* protein	Binding specificity	Ig class	V_H subgroup‡	I_{50}§
				ng/ml
T15	PC 1‖	IgA(κ)	V_{HIII}	21
T15 Fab				57
H8	PC 1	IgA(κ)	V_{HIII}	18
M603	PC 2	IgA(κ)	V_{HIII}	24
M511	PC 3	IgA(κ)	V_{HIII}	19
M167	PC 4	IgA(κ)	V_{HIII}	31
M167 Fab				121
CBPC 2	PC 1	IgA(κ)	V_{HIII}	27
ALPC 43	PC 5	IgG3	?	42
M460	DNP	IgA(κ)	V_{HI}	>10,000
M384	LPS	IgA(κ)	?	>10,000
J504	?	IgA	?	>10,000
Adj PC 22A	?	IgA	?	>10,000
M104	1,3 dextran	IgM(λ)	V_{HII}	>10,000
M173	?	IgG_{2a}(κ)	V_{HIII}	>10,000
M21	?	IgG_1(κ)	V_{HIII}	>10,000
J539	1,6 galactan	IgA(κ)	V_{HIII}	>10,000
x44	1,6 galactan	IgA(κ)	V_{HIII}	>10,000

* Myeloma proteins were purified except for J504 and Adj PC 22A where ascites fluid was used.
‡ From Barstad et al. (17).
§ I_{50}, concentration of protein which gives 50% inhibition of binding. Mean results of 2–3 experiments.
‖ Number designates type of binding specificity pattern for choline analogues (15, 18, 21).

PC-binding myeloma of C57BL/ka background (21), and on ALPC 43, an IgG3 protein arising in a BALB/c · AL/N IgC_H mouse. Each of the PC-binding myeloma proteins gave comparable inhibition of binding of [^{125}I]M167 to antibody-coated tubes indicating that serologically the cross-idiotypic determinant on each of these myeloma proteins was identical. Furthermore, pepsin Fab fragments from both T15 and M167 inhibited binding of [^{125}I]M167 to the idiotypic antiserum almost as efficiently as the unfragmented proteins (Table I). Control proteins such as normal mouse globulin and other, non-PC-binding myeloma proteins, including those in the same V_H subgroup as the PC-binding proteins, showed negligible reaction with the idiotypic antiserum. Thus, this antigenic determinant, designated V_H-PC, was found on all myelomas which bound PC, regardless of specificity, and appeared to be associated with variable region portions of the molecules rather than constant region or allotypic determinants.

Presence of the Cross-idiotypic Determinant in the Variable Region. Though present in T15 and M167 Fab fragments, the cross-idiotypic determinant appeared not to be located in the binding site, since the monovalent ligand, PC, failed to inhibit the binding of [^{125}I]M167 in the TBA (Fig. 2 left). This was corroborated by experiments which demonstrated that anti-V_H-PC could react with H8 in which the combining sites had been blocked by covalently bound PC groups. As shown in Fig. 2 (right) affinity labeled H8 gave no less inhibition of

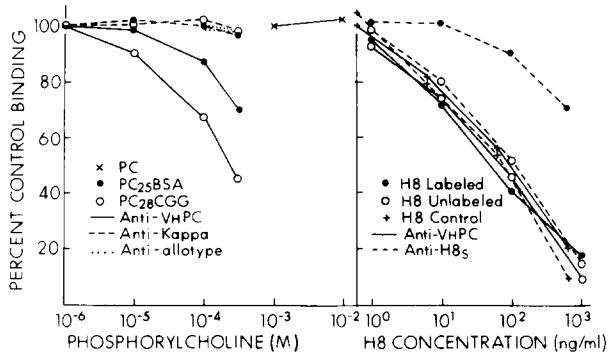

FIG. 2. Association of V_H-PC idiotype with the binding region. *Left:* PC, $PC_{25}BSA$, and $PC_{28}CGG$ were tested for the ability to inhibit the reactions between [^{125}I]M167 and Cl anti-H8 (anti-V_H-PC), anti-kappa and antiallotype. *Right:* Site-labeled and unlabeled T15 were tested for their ability to inhibit the binding of [^{125}I]M167 to Cl anti-H8 (anti V_H-PC) or [^{125}I]H8 to anti-$H8_s$.

binding of [^{125}I]M167 to anti-V_H-PC than did unlabeled H8. By contrast, affinity labeled H8 was a poor inhibitor of the binding of [^{125}I]H8 to antibodies (anti-$H8_s$) specific for combining site antigenic determinants (24). However, PC conjugated to BSA or CGG did inhibit the [^{125}I]M167 anti-H8 reaction by 30 and 55%, respectively. Neither of these conjugates influenced the binding of [^{125}I]M167 to anti-kappa or to antiallotypic sera. These data suggest that the determinant, though not directly in the PC-binding region, is nevertheless associated with the site so that steric blockage by the BSA or CGG carrier proteins occurred.

Chain Association of the Cross-idiotypic Determinant. To test for the possibility that the common idiotypic determinant described here was situated on the heavy chain, as the sequence data of Barstad et al. (17) might indicate, isolated heavy and light chains from four different proteins, H8, M511, M167 and M603, were tested in the inhibition assay for their ability to bind the idiotypic antiserum. As shown in Fig. 3, the inhibitory capacity of the H chains from each of the myeloma proteins (50% inhibition ~100 ng/ml) was comparable to that obtained with the intact proteins (50% inhibition ~25 ng/ml). On a molar basis, Ig was approximately five times more efficient as an inhibitor than H chains. However, this difference may be due to the bivalent nature of the whole Ig which should increase its inhibitory capacity. Light chains of these same proteins did not bind appreciably to the anti-V_H-PC idiotypic antibodies. It is unlikely that whole Ig or L-chain contamination of H chains could account for the inhibition observed with heavy chains. Chromatographic analysis showed no peak which could represent intact, unfractionated protein in the H-chain preparation and, as described in the Methods, less that 1% of L chains were detected in the H-chain preparation. More importantly, an idiotypic determinant which depends on an H-L interaction, the $H8_s$ idiotype (24), was not present in the H-chain preparations of H8 that were used in the experiments described here.

Cross-idiotypic Specificity Among Mouse Anti-PC Antibodies. Purified mouse IgM anti-PC antibodies were examined for the presence of the cross-idio-

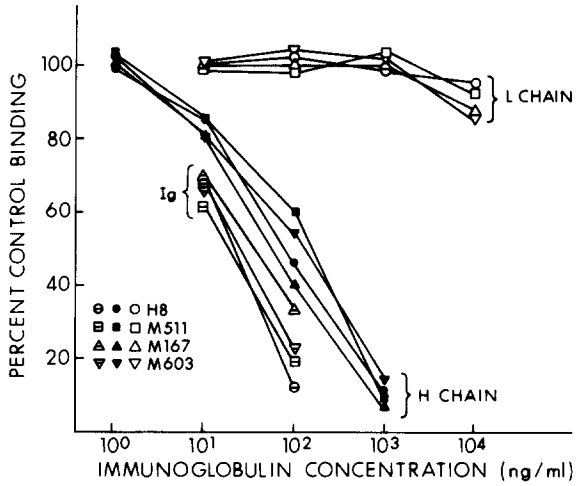

FIG. 3. Location of V_H-PC on the heavy chain. Unfractionated myeloma proteins and heavy and light chains derived from them were tested for inhibition of binding of [^{125}I]M167 to Cl anti-H8.

typic determinant. As demonstrated in Fig. 4, anti-PC antibodies obtained from mice representing each of the major IgC_H allotypic subgroups possessed the cross-idiotypic determinant common to the heavy chain of the PC-binding myeloma proteins (BALB/c and C58, a^1; C57BL/6, a^2; DBA/2, a^3; A and AKR, a^4; and CE, a^5). Moreover, since each antibody preparation gave comparable inhibition, the antigenic determinant on anti-PC antibodies from each of these strains was idiotypically identical to that on H8. In addition, this cross-idiotypic determinant was present on anti-PC antibody isolated from the sera (12, 13) of three different, immunized wild *Mus musculus*. It was not found on anti-PC antibodies of guinea pigs, rats, hamsters, and *Peromyscus* species. Thus, evidence is provided that heavy chain variable region portions of murine anti-PC antibodies, in addition to binding site determinants, e.g. $H8_s$, are preserved among different mouse strains.

Discussion

The present studies describe a cross-idiotypic determinant, V_H-PC, which is shared by PC-binding myeloma proteins that differ in binding specificity and have unrelated individual antigenic specificities. These immunoglobulins also differ in genetic origin, immunoglobulin class, and in the κ-light chain subgroup. Of particular importance is the finding that V_H-PC can be localized to the variable region of the heavy chain. This result supports the conclusions of Barstad and Rudikoff (17) that BALB/c PC-binding myeloma proteins and, by inference, antibodies to PC, have structurally similar heavy chain variable region(s). Indeed, as shown by the present study, induced IgM anti-PC antibodies isolated from genetically unrelated strains of mice possess the V_H-PC determinant.

There now exists three known variable region markers for murine immunoglobulins with specificity for PC (Table II). The first, T15, is a nonbinding site determinant which requires both heavy and light chains for full expression of its

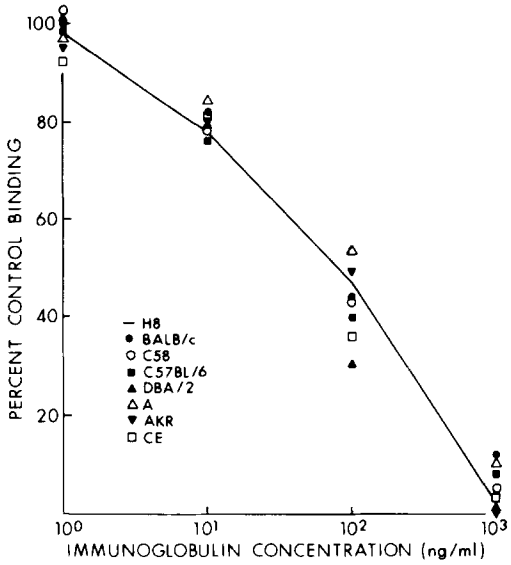

FIG. 4. Similarity of V_H-PC cross-idiotypic determinant among mouse anti-PC antibodies. Purified anti-PC antibodies inhibited the reaction between [^{125}I]M167 and Cl anti-H8.

TABLE II

Variable Region Antigenic Determinants on PC-Binding Immunoglobulins

Source of PC-binding immunoglobulin	Determinant*		
	T15	$H8_s$	V_H-PC
IgC_H allotype‡			
a^1	+ and −§	+	+
a^2	−	+	+
a^3	−	+	+
a^4	−	+	+
a^5	−	+	+
Myeloma protein ‖			
T15 group	+	+	+
M167	−	−	+
M603	−	−	+
M511	−	−	+

* T15 determinant, ref. 15 and 16; $H8_s$ determinant, ref. 13 and 24; V_H-PC, this paper.

‡ Anti-PC antibody found in mice representing designated allotype (22).

§ Some, but not all, strains positive (16).

‖ IgA(κ) myeloma proteins induced in BALB/c mice (IgC_H a^1).

antigenicity (14, 16, 24). Except for some possible discrepancies (29; Claflin and Davie, unpublished observations), this determinant is found principally on anti-PC antibodies or myelomas raised in mice of the a^1 IgC_H heavy chain linkage group (16) and does not correlate with binding site specificity (15). The second

determinant, $H8_s$, has been shown to lie in the binding site (24) and correlates precisely with binding specificity (12, 13). Therefore, antibodies raised in mice representative of all heavy chain linkage groups and myeloma proteins in the T15 group possess this determinant. The third determinant, V_H-PC, as shown in this paper, lies solely on the heavy chain and is found on all anti-PC immunoglobulins regardless of their specificity.

The latter determinant, V_H-PC, is clearly on a variable region of the heavy chain although its precise location is not determined. In other instances of cross-idiotypy, attempts to find activity on isolated H or L chains have been unsuccessful (4, 8, 9, see 30), and instead, have relied on structural studies to associate a cross-idiotypic determinant with a particular polypeptide chain (see 10, 31, 32). Recently, Thunberg and Kindt (4) have successfully employed heterologous recombinant molecules to localize an idiotypic determinant.

V_H-PC also appears loosely associated with the combining site, since PC-protein conjugates, but not PC, alter the binding of the idiotypic antibody to V_H-PC. Apparently the V_H-PC determinant is not in the precise region of PC binding, but is sufficiently close to it so that steric blockage occurs with portions of the protein conjugate which are adjacent to the PC-linkage. While this approach has obvious limitations, it can be used along with structural data to indicate potential locations of V_H-PC. In fact, considerable information concerning the tertiary structure of one of the PC-binding proteins used in the present study has been generated by X-ray crystallographic studies (33). In M603, PC is bound at one end of the Fab in a large cleft, the entire surface of which is composed of five hypervariable regions. PC, even though it interacts with at least four of these hypervariable regions, occupies only a small portion of the cleft. Thus, PC binding leaves most of the surface of the hypervariable loops "free". On the other hand, PC-protein binding would likely cover all heavy chain hypervariable regions in the cleft. Of the possible candidates for V_H-PC location, the first hypervariable region of the heavy chain appears the most likely, since it, in contrast to the second and third (S. Rudikoff, personal communication), is the only one which is structurally identical in all the PC-binding proteins so far sequenced and which is not found in the heavy chain of other immunoglobulins (17).

The present findings support an ever growing body of literature which indicates that antibody molecules with similar specificity are similar structurally and/or antigenically. Among human IgM proteins with anti-γ-globulin activity (6, 10, 11) and among rabbit and mouse antistreptococcal carbohydrate antibodies (4, 5, 7, 31), cross-idiotypic determinants are more strongly associated with the antibody combining site and/or hypervariable regions than with other portions of the variable regions. These conclusions are based on a variety of data, including similarity in specificity for a particular antigen, antigen or hapten inhibition of binding to idiotype and sequence similarity of hypervariable regions in immunoglobulins in which the remainder of the variable region is virtually identical to a number of other idiotypically unrelated proteins. As exemplified by the PC system, structural studies have shown that a particular V_{HIII} heavy chain subtype is present in three different BALB/c myeloma proteins (17) and in one protein of C57BL origin (21). Since the presence of the V_H-PC determinant

correlated with the occurrence of the PC V_{HIII} subtype in these sequenced proteins, it is probable that the heavy chains of each of the other proteins (MOPC 511 and ALPC 43) belong to this V_{HIII} subtype as well. Indeed, one could predict that a prerequisite for PC-binding activity in all mice, regardless of genetic background, is this PC V_{HIII} subtype. Additional sequence data will be required to determine the extent to which this heavy chain has been conserved in anti-PC antibodies.

Finally, it should be emphasized that all immunoglobulins undoubtedly possess multiple "idiotypic" determinants. While we have demonstrated three such determinants on anti-PC immunoglobulins, there are probably others on the same molecules. The crucial point in terms of using these antigenic determinants as structural or clonal probes, is that each, because of different structural localization, may have completely different distributions among other immunoglobulins. Thus, distributions of the three idiotypic markers among anti-PC molecules of mice are dramatically different and could lead to different conclusions if used as genetic markers without knowledge of their structural correlates. It should be obvious that most, if not all, idiotypic antisera probably contain antibodies to several determinants. Therefore, studies which use idiotypic antisera as genetic or structural probes are most informative if the number and location of the idiotypic determinants measured are known. Without the structural correlates, the use of idiotypes may only confuse our understanding of antibody interrelationships; with more precise definition of the idiotypic determinants, they may become our most useful probes.

Summary

Seven mouse myeloma proteins with specificity for phosphorylcholine (PC) were found to share a common antigenic determinant. This group of proteins contained members which differed in genetic origin, heavy chain class, κ-chain subgroup, individual antigenic determinants and specificity for choline analogues. The cross-idiotypic determinant, V_H-PC, was antigenically similar in each of the proteins and was associated with the variable portion of the heavy chain in the region of the antibody combining site. Further studies showed that an indistinguishable determinant was present on IgM anti-PC antibodies isolated from all strains of mice tested regardless of histocompatibility or heavy chain allotype. In view of the finding that this cross-idiotypic determinant was not found on antibodies or myeloma proteins which lacked specificity for PC, the data strongly suggest that a particular heavy chain variable region has been preserved in all mouse antibodies with specificity for PC.

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