ANTI-PHOSPHOCHOLINE HYBRIDOMA ANTIBODIES

II. Functional Analysis of Binding Sites within Three Antibody Families*

By CATHERINE M. ANDRES, ANNE MADDALENA, SUSAN HUDAK, N. MARTIN YOUNG, and J. LATHAM CLAFLIN

From the Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109; and the Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A0R6

Anti-phosphocholine (PC)[1] antibodies raised in the serum response to PC-antigens segregate into three main families on the basis of differential recognition by anti-idiotypic (Id) antibodies. One family shares public Id determinants found on a group of nearby identical PC-binding myeloma proteins (MP), e.g., TEPC 15, HOPC 8, S107. Another family possesses public determinants common to two other anti-PC MP, MOPC 511 and MOPC 167, whereas the third contains public Id shared by another two anti-PC MP, McPC 603 and W3207 (1–4). Recent studies (5) on hybridoma proteins (HP) generated from six different strains of mice immunized with two different PC-antigens, *Streptococcus pneumoniae* (R36A) and PC-protein, have confirmed the presence of these three families and their dominance in the immune response. Structural and biochemical studies on hybridoma antibodies have shown that each of the three families uses a distinct V region for its L chain (1, 6).[2] The L chains in the T15 family belong to the VK-22 isotype, those in the M511 family use VK-24 L chains, and those in the M603 family are VK-8 L chains. By contrast, the VH all fall into the same V region isotype, VH-4. Thus, these HP families show conservation that parallels that seen in serum anti-PC antibody families (7, 8).

In spite of this observed structural similarity, idiotypic and isoelectric focusing studies revealed heterogeneity within a family (2–4, 9, 10). Our initial experiments on HP (5) suggested that some of the structural heterogeneity occurred in the binding site. To evaluate the functional significance of this heterogeneity, we performed more detailed studies on the binding activity of the HP using three different PC-antigens (carrier effects) and a variety of choline (C) analogues (hapten effects). We have confirmed our previous observations that each family can be distinguished from the others. In addition, we have detected binding-site heterogeneity for hapten within members of the M511 and M603 families but not among members of the T15 family, even though the latter are derived from several strains and have known V_H and V_L

* Supported by grant AI 12533 from the National Institutes of Health. Catherine M. Andres was supported in part by National Institutes of Health Clinical Education Grant CA 19762. Anne Maddalena was supported in part by an F. G. Novy Fellowship.

[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; C, choline; HP, hybridoma protein; Id, idiotype; IEF, isoelectric focusing; MP, myeloma protein; PBS-A, phosphate-buffered saline, pH 7.4, containing 0.04% sodium azide; PC, phosphocholine; SRIA, solid-phase radioimmunoassay.

[2] S. Clarke, L. Claflin, M. Potter, and S. Rudikoff. Manuscript in preparation.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/11/1584/15 $1.00
Volume 154 November 1981 1584-1598

differences. A third observation is the existence of differential reactivity for carrier determinants among HP and MP that exhibit indistinguishable hapten-binding profiles. These results are discussed in terms of structure-function relationships and the biological importance of functional heterogeneity within antibody families arising in the same response.

Materials and Methods

Myeloma and Hybridoma Proteins. The PC-BMP, T15, H8, M603, W3207, M511, and M167 were purified from ascites fluid by affinity chromatography on PC-Sepharose, as previously described (1). Anti-PC HP were generated by cell fusion with the nonsecreting cell lines SP2/0-Ag14 (11) (fusion numbers ≤116) or X63-Ag8.653 (12) (fusion numbers ≥120). Spleen cells were obtained from mice immunized with 10^8 *Streptococcus pneumoniae* (R36A) (fusion numbers ≤120) or with 100–200 μg PC-keyhole limpet hemocyanin or bovine gamma globulin (BGG) (fusion number ≥130 and 116), as previously described (5). Anti-PC hybridomas were propagated in Iscove's hypoxanthine-aminopterin-thymidine, subcloned in soft agar over thymocyte feeder layers (13), and stored frozen. Selected hybridomas were grown in ascites in pristane-primed H-2-compatible or irradiated mice. The anti-PC HP were purified by affinity chromatography, as described above. All HP were found to contain a single molecular species as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) in polyacrylamide gels (1). HP are designated by a three-component number m.n.o., where m refers to the fusion number, n refers to the clone number, and o refers to the subclone number.

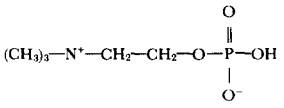
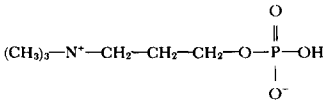
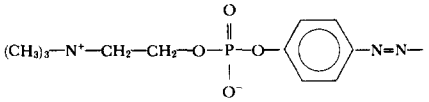
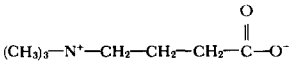
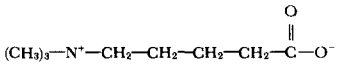
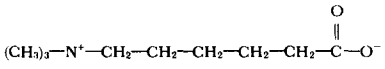
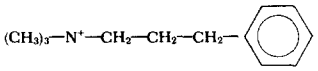
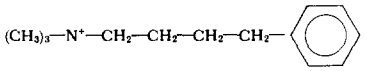
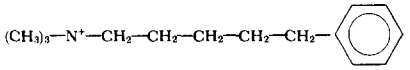
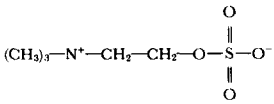
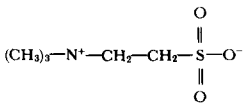
Haptens and PC-Antigens. PC and L-α-glycerophosphocholine (GPC) were purchased from Sigma Chemical Co., St. Louis, Mo. 3-(*p*-azophenylphosphocholine)-N-acetyl-L-tyrosylglycylglycine (YGG) Boc hydrazide (PC-Y) was purchased from Biosearch, San Rafael, Calif. All the remaining haptens were synthesized from commercial precursors, using C, H, N elemental analysis as the criterion of purity. These compounds have been described previously (14), and they are all listed in Table I. PC-BGG was prepared as previously described (2). Soluble PC-containing antigens were prepared from extracts of R36A and *Proteus morganii* (Potter) (15, 4). Briefly, washed R36A bacterial cells at concentrations ≥10^9/ml were heated at 60°C for 30–40 min. After centrifugation at 5,000 *g*, the cell-free lysate was precipitated three times with 80% ethanol in water and then dissolved in phosphate-buffered saline containing 0.04% sodium azide (PBS-A). Washed *P. morganii* cells were extracted with 0.05% SDS, 0.015 M sodium citrate, and 0.15 M NaCl at 56°C for 60 min. The cell-free supernatant was exhaustively dialyzed against PBS-A. These antigens still contained protein complexed with the PC-antigen that made possible radiolabeling with iodine. Both the R36A and *P. morganii* extracts contained C (4) and gave strong precipitin reactions with T15 and M603, respectively.

Antigen-binding of Anti-PC Hybridoma Antibodies. The binding pattern of each HP for the C analogues listed in Table I was assessed in a competitive solid-phase radioimmunoassay (SRIA). A comparison was made of the molar concentration of the C analogues required to inhibit the reaction between antibody-coated plates and ^{125}I-labeled PC-BGG (5), R36A, or *P. morganii* extracts. The relative binding efficiency was expressed as the ratio of the molar concentration of C analogue giving 50% inhibition (I_{50}) to the molar concentration of PC at I_{50}. A stock solution at 0.1 M was made for each analogue in PBS, pH 7.4, and used for all experiments. This proved a useful and reproducible assay for accurately measuring and comparing hapten-binding activities of anti-PC antibodies.

The ability of each anti-PC antibody to bind PC attached to different carriers was measured in direct titration SRIA. Briefly, dilutions (100–0.1 μg/ml) of each HP and MP were used to coat duplicate wells in polyvinylchloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). After overnight incubation, washing with PBS-A, and brief incubation in PBS-A containing 1% BSA, 10,000 cpm of radioiodinated PC-BGG, R36A, or *P. morganii* extracts were added, with one antigen for each set of dilutions. Maximum binding was taken from the plateau portion of the curve that occurred between 100 to 10 μg/ml.

TABLE I

Hapten Analogues

Compound	Figure symbol, abbreviation	Structure
Phosphocholine	■—■, PC	
3-Phosphopropyl TMA*	●—●, PPTMA	
3-(*p*-azophenyl PC)-*N*-acetyl-YGG Boc hydrazide	○—○, PC-Y	 YGG Boc hydrazide
3-Carboxypropyl TMA‡	●—●, 3COOH	
4-Carboxybutyl TMA§	★—★, 4COOH	
5-Carboxypentyl TMA§	⭘—⭘, 5COOH	
3-Phenylpropyl TMA§	●·····●, 3 ϕ	
4-Phenylbutyl TMA§	★·····★, 4 ϕ	
5-Phenylpentyl TMA§	⭘····⭘, 5 ϕ	
o-Choline sulfate	■—·—■, OCS	
2-(TMA) Ethanesulfonic acid	TES	

* TMA = trimethylammonium.
‡ Iodide salt.
§ Bromide salts.

Results

Anti-PC Antibodies. Table II summarizes the origin and characteristics of the monoclonal anti-PC antibodies used in this study. Many of these MP and HP have been described previously (5). All the MP are IgA and arose in BALB/c or CBB22, a BALB/c congenic strain carrying the C57BL allotype at the *Igh* locus. The HP

TABLE II
Anti-PC Hybridoma and Myeloma Proteins

HP or MP	Mouse strain	Igh	Ig Class	^{125}I-labeled antigen*		
				PC-BGG	*S. pneumoniae*	*P. morganii*
The T15 Family						
T15	BALB/c	a	IgA	4,220	1,090	220
H8	BALB/c	a	IgA	4,068	980	400
134.5B11	BALB.G	a	IgG_3	5,130	1,011‖	193‖
59.6C5.1	BAB14	a/b	IgG_3	5,420	1,110	1,750
C3	CBB22	b	IgA	4,690	830	80
22.1A4‡	AKR	d	IgM	4,780	680	280
101.8E4.4	CBA	j	IgM	4,660	890	300
140.7C6.2	CBA	j	IgM	5,060	1,000	350
100.6G2.1	CBA	j	IgG_2	5,050	1,140	1,530
140.3D9.2§,.1C2.2	CBA	j	IgG_2	5,180	1,030	2,650
103.1C9.1‡	PL	j	IgG_2	5,320	1,200	1,550
The M511/M167 Family						
M511	BALB/c	a	IgA	4,490	460	0
M167	BALB/c	a	IgA	5,100	640	0
137.2D3	C57L	a	IgM	ND¶	ND	ND
137.7C9.1§, .5G6.1	C57L	a	IgG_1	2,300	160	0
100.1C11.5	CBA	j	IgM	3,250	330	0
101.3C2.2	CBA	j	IgM	4,100	410	0
101.6G6.2,§.3G8.4, .6E5.1,.6F6.4	CBA	j	IgM	4,830	190	150
120.2E5.2	CBA	j	IgM	ND	400	0
The M603 Family						
M603	BALB/c	a	IgA	2,950	490	3,350
W3207	BALB/c	a	IgA	3,580	530	500
55.6F3.4,.7C8.4§	BALB/c	a	IgM	3,210	300	480
116.5F3.2	BALB.G	a	IgG_1	4,540	230	0
131.3B8	BALB.G	a	IgG_3	ND	ND	ND
137.6F2.1	C57L	a	IgG_2	ND	ND	ND
100.6F9.1,.6G2.2§	CBA	j	IgM	3,780	550	3,270

* Cpm of antigen bound by antibody-coated wells. Antibody concentrations of 10 μg/ml (vs. PG-BGG) or 100 μg/ml (vs. *S. pneumoniae* or *P. morganii*) were used to coat duplicate wells. 10,000 cpm of radiolabeled antigen were added to each well.

‡ Carries Igk-PC-A marker; all other HP in this family are Igk-PC-B.

§ HP on the same line arose in the same fusion and were identical by idiotype and binding studies. Results of antigen binding are reported for the first HP listed; all others in the group were similar.

‖ Results obtained with 10 μg/ml of antibody.

¶ Not done.

represent all the major serum isotypes except IgA and come from seven different strains. Idiotyping of HP consistently yielded the three major groups or families shown, regardless of strain. IEF of isolated L chains showed that within a family, the L chains co-isofocused (except for HP116.5F3.2, which has been tentatively assigned to the M603 family, and for electrophoretic shifts resulting from genetic markers in the T15 family) (5). Sequence analysis of the MP has shown that the L chains within a family are either identical or strikingly similar, but between families they belong to three different VK isotypes (16, 17). Sequence analysis of representative HP reveals exactly the same pattern (6).[2] Thus, in this instance there is an absolute correlation

between an IEF pattern and a VK isotype. The H chains of all the MP and HP so far sequenced belong to the VH-4 isotype.

In published and unpublished studies, we and others have been able to subdivide members of family idiotypically. For example, T15, C3, HP103.1C9.1, and HP140.1C2.2 can be distinguished from each other by private anti-idiotypic antisera (18, 19). Similar findings have been made for M511, M167, HP101.3C2.2, HP137.5G6.1, and HP101.6G6.2 in the M511/M167 family (20, 21) and for M603, W3207, HP55.6F3.4, HP116.5F3.2, and HP100.6F9.1 in the M603 family (S. Hudak, and L. Claflin, unpublished data). The bases for these idiotypic differences are currently unknown, but they do provide direct evidence for structural diversity within a family.

Binding Site Specificity: Carrier Effects. Anti-PC antibodies were first tested for their ability to bind three different PC-antigens, PC-BGG, and the two polysaccharide antigens isolated from *S. pneumoniae* and *P. morganii.* The results compiled from four experiments are summarized in Table II. All MP and HP bound the hapten-protein conjugate about equally well even though most of the HP were derived from R36A immunized mice, i.e., fusion number $\leq$120, except 116. The binding to R36A was more variable, particularly within the M511 and M603 families. The T15 HP consistently bound more of this antigen than did HP of the M511 or M603 families. No correlation was observed between carrier specificity and antigen used to prime the spleen cell donor. Binding to *P. morganii* occurred only among T15 and M603 HP and MP. Some of these antibodies bound *P. morganii* well (1,530–3,350 cpm), whereas others reacted poorly (80–500 cpm). One M603 HP, HP116.5F3.2, did not bind *P. morganii* at all. No strain or Ig class effects were seen except among the T15 family, where IgA or IgM HP were all weak binders and the IgG were much stronger binders. This was not related to the polymeric nature of the antibody because some strongly binding IgM and IgA HP were observed among M603 HP.

Binding Site Specificity: Hapten Effects. To obtain additional information about binding site diversity, the binding reactivity of each anti-PC antibody was measured for a large number of analogues of C. These analogues were all derivatives of C (Table I) in the form $(CH_3)_3N^+(CH_2)_n$-R. They all contained an unmodified, positively charged trimethylammonium group that is essential for binding (22) and a charged or uncharged R group at various distances from the quaternary nitrogen. This length variation was accomplished with $-CH_2$-residues. The substituents originally tested were phenyl, hydroxyl, and carboxylate, in addition to phosphate, sulfate, and sulfonate. The results obtained with the hydroxyl series and the compounds in which the distal substituents were either very close together or far apart are not reported because they gave negligible inhibition with all HP. The inhibition results obtained with more informative C analogues and representative members of all three antibody families are depicted in Figs. 1–3. A comparison of the inhibitory capacity relative to PC is tabulated in Fig. 4.

Nonspecific effects of the C analogues on protein interactions can be discounted because of the range of activity the analogues showed with different antibodies. In an independent control, the C analogues at 0.001 M were unable to inhibit >20% of the binding of a monoclonal anti-TNP antibody to TNP-BGG.

The T15 Family. The fine specificity of all the T15 proteins listed in Table II were examined. Representative examples are shown in Fig. 1. The MP and HP presented

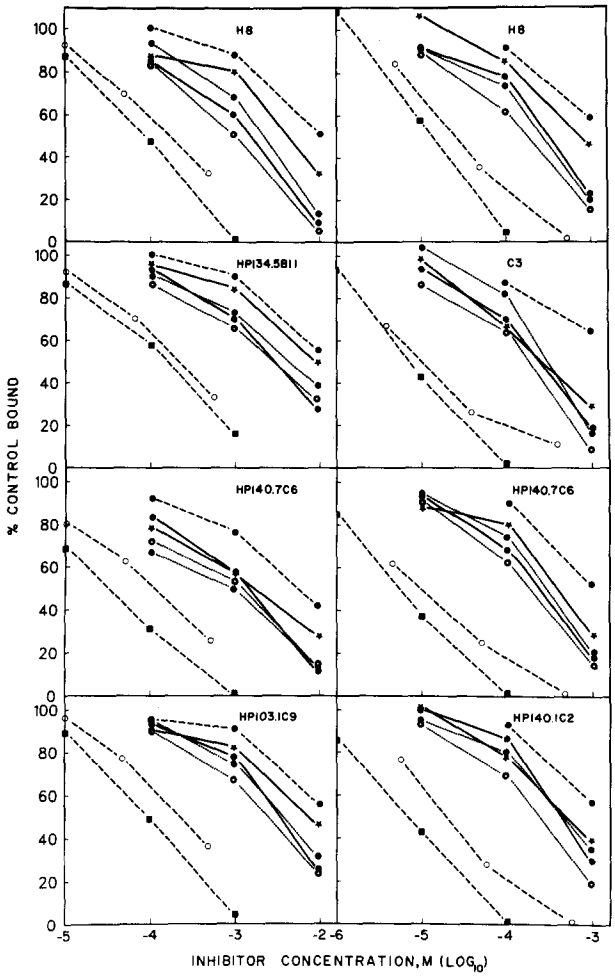


FIG. 1. Binding site profiles at anti-PC HP in the T15 family. Binding was measured in a competitive RIA between HP and ^{125}I-PC-BGG (left panels) or ^{125}I-*S. pneumoniae* polysaccharide (right panels). Inhibitors and symbols are listed in Table I.

are indicative of the types of diversity that exist among the T15 HP. These consist of the following: (*a*) CH isotype: included are two or more proteins of each of the following classes: IgA, IgG_{2b}, IgG_3, and IgM. (*b*) CH allotype: the proteins arose in mice with these Igh allotypes: a, b, a/b, d, and j. (*c*) V_K polymorphism: the T15 L chains have the isofocusing phenotype of Igk-PC-A or Igk-PC-B. (*d*) Idiotype: anti-idiotypic sera made against selected HP react with the T15 family of antibodies in groups according to individual, allotype, and family specificities (A. Maddalena and J. L. Claflin, unpublished observations). (*e*) Sequence: among the MP, V_H substitutions are known. In the D region, H0PC8 is GLN_{99}, whereas T15 is SER_{99} (16); T15 is PRO_{14}, GLY_{16}, PRO_{40}, ARG_{44}, ALA_{108}, whereas C3 is SER, ARG, ALA, GLY, THR, respectively (17). In addition, the donors for fusions were immunized with R36A or PC-protein.

Among all the MP and HP of this family, the fine specificities are as similar as

replicate trials of one protein (Fig. 4). Whether the competing antigen was PC-BGG (Fig. 1, left panel, 4) or R36A (Fig. 1, right panel, 4), the characteristic binding order of the analogues is PC > PC-Y ≫ 5ϕ ≥ 3COOH ≥ 3ϕ ≥ 4COOH > 3PPTMA. Those HP that can bind *P. morganii* do so with the same fine specificity with which they bind the other two antigens (data not shown).

M603 Family. The nine M603 MP and HP were derived from four different strains, one of which was congenic to BALB/c. They belonged to all the major CH isotypes. All the M603 MP and HP except one exhibited the same binding pattern for the C haptens, PC > PC-Y = 3COOH ≫ 4COOH = OCS ≥ 3PPTMA > 5ϕ. A typical result is shown in Fig. 2. Repetition of the experiment on three separate occasions did not reveal any significant differences among these antibodies (Fig. 4). Of particular interest are the findings that: (*a*) the inhibition profiles for M603 and HP100.6F9, which bind *P. morganii*, and W3207 and HP55.7C8, which do not bind this antigen well are indistinguishable; and (*b*) The antibodies were the same when they were tested with either PC-BGG or *P. morganii* (Figs. 2 and 4). The exceptional pattern was seen with HP116.5F3, whose molar ratios (vs. PC) at I_{50} were PC-Y, 0.0022; 3PPTMA, 0.69; 4COOH, 0.83; PC, 1; 3COOH, 1.7; 5ϕ, 3.3; and OCS, 17.

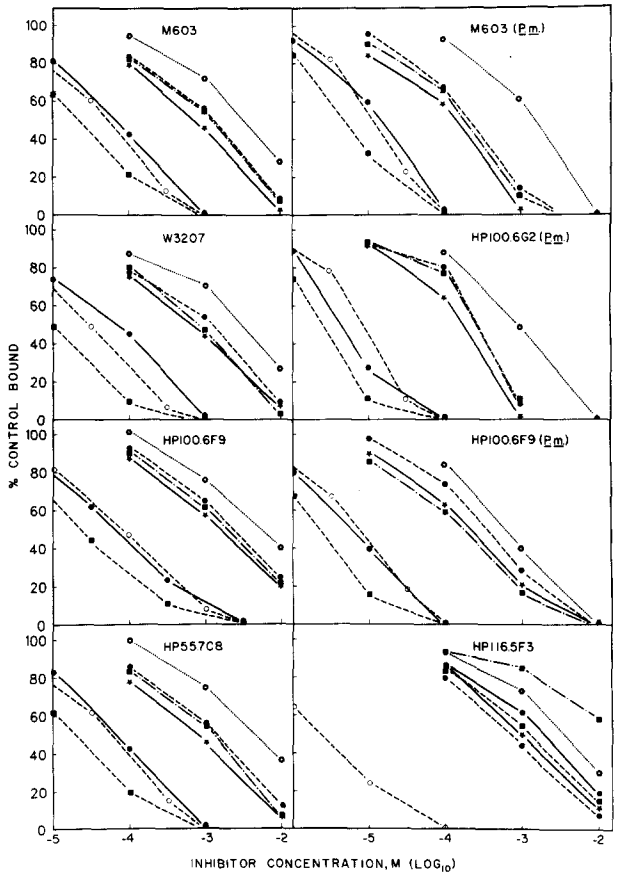


FIG. 2. Binding site profiles of anti-PC HP in the M603 family. Binding was assessed as described in Fig. 1, using ^{125}I-PC-BGG or ^{125}I-extract of *P. morganii* (panels having *P. m.* after name of HP).

This antibody was derived from a PC-protein-immunized animal and showed the strongest binding with the analogue closest in structure to the hapten on the immunizing antigen, PC-Y. By contrast, HP137.6F2.1, which also arose in a C57L mouse injected with PC-protein, possessed a binding pattern identical to the other HP. All HP and MP, except HP116.5F3, had L chains that co-focused with M603. No correlation of structure and fine specificity was observed. For instance, W3207 and M603 exhibit differences in H chain CDR 2 and 3 and some differences in their L chains (16, 23), but this is not reflected in their binding preference for the C analogues.

The M511 Family. The inhibition profiles obtained with these HP contrast sharply with those in the T15 and M603 families (Figs. 3 and 4), even though they came from the same strains of mice. No two patterns are the same unless the HP came from the same fusion, e.g., 101, 137. One group of four HP from fusion 101, prototyped by HP101.6G6.2, all exhibited the same inhibition pattern (▲ and ▼ in Fig. 4). They also share private idiotypes (21). The two BALB/c MP M511 and M167, whose structures are similar but not identical, can be readily distinguished from each other. Of particular interest is the finding that one BALB/c antibody (M511) and one CBA antibody (HP100.1C11.5) have quite similar binding orders for the C analogues (★ and ☆ in Fig. 4).

For all M511 HP and MP, PC and PC-Y were consistently excellent inhibitors. In the carboxylate series $(CH_3)_3N^+(CH_2)_nCOO^-$, the inhibitory activity tended to increase as n decreased to 3, although for one HP, HP101.3C2.2, the 4COOH hapten was the best inhibitor. The n=3 carboxylate compound compares in size with PC. Substitution of a phenyl group for the negatively charged phosphate or carboxylate group led to variable results. With four of the six antibodies, the phenyl series failed to give significant inhibition; M511 and HP100.1C11.5, however, showed excellent

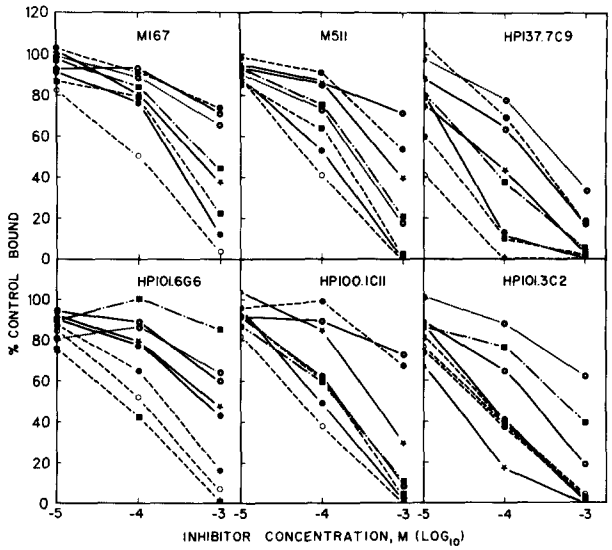


FIG. 3. Binding site profiles of anti-PC HP in the M511 family. Binding was assessed as described in Fig. 1 with ^{125}I-PC-BGG.

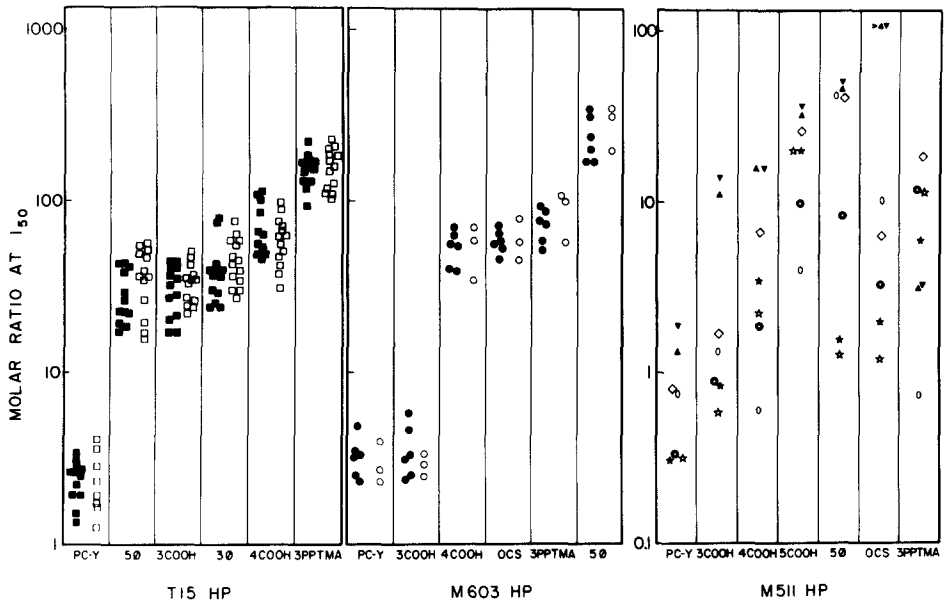


FIG. 4. Summary of binding specificity profiles for anti-PC antibodies in the T15, M603, and M511 families. Data are expressed as a ratio of C analogue (listed) to PC at I_{50}. The ratio for any one compound with an HP showed a 1.2- to 2.3-fold variation between experiments. Abbreviations for analogues are given in Table I. Left two panels: ■, T15 Id+ and ●, M603 Id+ HP and MP tested against ^{125}I-PC-BGG; □, T15 Id+ HP and MP tested against ^{125}I-R36A. Results from single experiments or mean results obtained from two to five assays with each HP and MP listed in Table II, except HP116.5F3.2, are presented. ○, M603 and the M603 Id+ HP 100.6F9.1 and 100.6G2.2 tested against ^{125}I-*P. morganii*. Right panel: note scale change; ✪, M167; ★, M511; ☆, HP 100.1C11.5; ○, HP 101.3C2.2; ▲, HP 101.6G6.2; ▼, HP 101.3G8.4 and ◇, HP 137.7C9 tested against ^{125}I-PC-BGG. Mean results from four to five experiments.

reactivity with the n=5 compound. Each antibody showed a unique pattern of reactivity with OCS and 3PPTMA.

Discussion

Multiple approaches have been used to examine diversification processes of antibodies having the same specificity. These include idiotypy, model building, measurement of binding activity, and complete amino acid sequencing. We have emphasized the latter two approaches becasuse we are primarily interested in how structural differences translate into functional diversity. Idiotypic studies, for example, have aided in measuring the extent of structural diversity, but it does not necessarily follow that a structural change leads to a new binding site. Structural differences are already known to exist among anti-PC MP and HP and additional ones are inferred from IEF studies and the presence of private idiotypes (18–21). To understand the significance of this heterogeneity, especially within a family, we attempted to obtain a "fingerprint" of the binding site both in its reactivity for hapten and for hapten plus carrier. For the former, we used hapten inhibition of binding to ligand, in this case PC linked to three different carriers. Leon and Young (24) successfully used this approach first to recognize the specificity of the anti-PC MP and to identify functionally different classes of anti-PC antibodies. Others have used hapten inhibition studies

to identify one group of antibodies in a much larger population (25) or to subdivide HP having a particular specificity into distinct functional groups (26, 27). To assess carrier effects, we have tested the ability of each MP or HP to bind the artificial antigen PC-BGG and two natural PC-antigens, *S. pneumoniae* and *P. morganii*. In each instance the haptenic moiety is constant. However, the linkages as well as the adjacent carrier determinants are quite different. In PC-protein conjugates, PC is phosphodiester linked to a phenyl residue para to its diazo linkage primarily to tyrosines in the protein (28). In *Pneumococcus*, PC substituents are phosphodiester linked to unacetylated galactosamine residues in a repeat tetrasaccharide unit of C substance (29). The linkage of PC to cell wall constituents of *P. morganii* is not known, but because the composition of a Gram-negative cell well differs from a Gram-positive cell wall and because *P. morganii* exhibits different reactivity with PC-BMP and antibodies (4, 16), we can infer that the structure of its PC-antigen is different.

Each family of antibodies displays its own unique features and for that reason they will be considered separately.

The T15 Family. The various MP and HP of the T15 family all exhibit within the limits of the assay system the same fine specificity, even though we know from idiotypic and structural studies that they differ in their V regions. That pattern is characterized by the position of PC and PC-Y as the best inhibitors of each hapten-carrier complex, PC-BGG, R36A, and *P. morganii*. Analogues with substitutions for the phosphate group or with various chain lengths are 10–200 times less effective based on molar concentrations at I_{50}. Thus, when the phosphate group is spaced further away from the TMA group, e.g., 3PPTMA, or replaced with another group (hydrophobic or ionic), binding is measurable but significantly weaker. These results suggest important binding site interactions between T15 antibodies and PC at both the trimethylammonium determinant and the phosphate determinant. This would be predicted by analogy to the crystal structure of M603 (30, 31).

The fact that PC is the best inhibitor, regardless of the immunogen used to generate the T15 HP, indicates that interaction with the PC moiety in the ^{125}I-PC-BGG conjugate does not depend on the diazophenyl bridge or adjacent amino acids. This is supported by the activity of PC-Y, a compound that is always less effective than PC, requiring one to four times the concentration of PC to achieve comparable inhibition. Its structure has the same PC-phenyldiazo linkage that exists in PC-protein conjugates. Furthermore, the fine specificity of all T15 MP and HP is the same regardless of whether they are tested against PC-BGG, the pneumococcal, or, where possible, the proteus antigen. Thus, there exists a remarkable commonality in the T15 binding sites for PC. Differences are seen only in the ability to bind *P. morganii*. Thus, carrier specificity is independent of hapten specificity and probably reflects amino acid substitutions in CDR positions that interact with moieties of the carrier.

The M603 Family. Like T15 HP, all M603 HP and MP (except HP116.5F3), have a characteristic fine specificity profile for the C analogues, but it is different from the T15 profile. M603 HP display a greater relative affinity for the n=3 carboxylate derivative than do T15 HP, and 3COOH along with PC and PC-Y are the best inhibitors. M603 HP have a low affinity for the n=5 phenyl derivative in contrast to the T15 family. However, these HP resemble T15 HP in showing the strongest reactivity for PC, regardless of the origin of the HP or the ligand against which the fine specificity is measured. All the HP react with more than one carrier. A special

feature of some M603 HP is their high activity for *P. morganii*, yet there is no apparent relationship between hapten and carrier specificity. Thus, just like T15 HP, M603 HP share a common hapten-binding site but they display different reactivities for carrier determinants.

HP116.5F3 is an exceptional HP. It has the greatest relative activity for PC-Y and binds PC 500-fold less well and no better than other analogues. Thus, the antibody displays strong anti-bridge activity. Sequence analysis of this protein will prove extremely interesting because its L chain does not co-focus with M603 L chain, but it does contain the V_H-PC Id that appears to identify the VH-4 isotype (32). Does this antibody contain functionally important somatic mutations, different J segments, or an unusual DH? Or is it encoded by different members of the VH-4 or VK-8 (M603L) gene family?

The M511 Family. Unlike T15 and M603 HP, the M511 antibodies exhibit extreme binding site diversity. This is reflected not only in their binding to the hapten analogues but also in their level of reactivity for different PC-carrier complexes. For example, all bind PC-BGG about equally well, but they vary in their ability to bind R36A, and none bind *P. morganii* significantly. This was not entirely unexpected because we had previously shown (5) with a more limited number of C analogues that binding site diversity occurred in M511 HP. Examination of the structure of M511 and M167, for which complete H and L sequences are available, shows that these two proteins differ at multiple residues in H chain CDR 2 and 3. Two in CDR 2 are immediately adjacent to contact residues and one in CDR 3 at 100D is a contact residue for PC, at least in M603 Fab. Evaluation of these structural changes does not reveal a simple explanation for the disparate specificity profiles of M167 and M511. The L chains of M511 and M167 differ in six positions but these are all in the framework (33, 34). Because of the amount of structural variability, more sequences will be required before a picture emerges. However, it seems probable that a much greater degree of flexibility exists in the contour of the sites in various M511 antibodies than occurs in T15 and M603 HP. Model building exercises may permit a test of this hypothesis.

Do the existing structural data support the concept of "fixed" site for PC and a variable site for carrier? Two observations are pertinent. First, PC antigens exist in nature as the same hapten coupled to widely different carriers, i.e., microbial cell wall constituents. Second, the site for contact with PC-antigens, at least in the X-ray diffraction model of M603, is a large surface of CDR residues on one end of the Fab that extends into the molecule, forming a deep pocket (30). Thus, one could operationally distinguish between the pocket that interacts with PC and the outer surfaces that might interact with carrier determinants.

One could account for a fixed pocket by preservation of important charge interactions for PC. In fact, all anti-PC antibodies use a VH-4 heavy chain (34), and from analysis of the M603 X-ray structure (30, 31), it is clear that the major contact residues are found in the H chain. They are located in the pocket at positions 33 and 35 in CDR 1, positions 52 and 58 in CDR 2, and position 100 D in CDR 3. They are the same in all T15 and M603 antibodies sequenced to date (6, 16)[2] as are adjacent regions that could contribute van der Waals interactions with PC and aid in maintaining a regular beta-pleated sheet structure (28). Those regions in the H chain CDR displaying the greatest variability can be excluded as contact residues for PC

but should be considered important in carrier reactivity. From the sequence data it is clear that the greatest variability in H chains occurs in CDR 3, principally in the DH region. Other regions of significant but less extensive variability arise in the central portion of CDR 2. Again, examination of the three-dimensional model of M603 is revealing; these portions of the CDR 2 and 3 would be available to interact with carrier determinants. As an example, M603 and W3207 both have the same fine specificity for the C analogues, yet they differ markedly in their binding of *P. morganii*. All the contact residues for PC are preserved in both antibodies, but striking differences occur in DH and the central portion of CDR 2.

How does one account for two different pockets for PC, one in T15 antibodies and another in M603 antibodies? Even without sequence data, one could suggest from the association of VK group with family that the L chain directly or indirectly determines the nature of the fine specificity pattern. Preliminary information obtained from heterologous H/L chain recombinants between H8, T15, M603, and W3207 support this hypothesis.[3] Clear definition of its role in PC and carrier recognition, as well as defining the contribution of CDR regions, will require more complete sequences of members in T15 and M603 families. Additional information should also be gained from chain recombination experiments with the HP, and as stated, these are in progress.

The genetic implications of a conserved and a variable component in an anti-PC site are intriguing. Evolution has apparently favored development of at least two common and fixed pockets for PC. The need for a common pocket undoubtedly reflects the nature of PC-antigens in different environmental pathogens. Perhaps the ubiquitous distribution of PC in different organisms or the prevalence of certain PC-containing antigens led to the evolution of two important types of sites for PC. The high concentration of T15 Id^+ anti-PC antibodies in normal serum (36) and the high frequency of T15 Id^+ precursor B cells (9) support this hypothesis. Levels of M603 Id^+ antibodies and precursors have not been measured in normal mice. However, these are the dominant antibodies after immunization with *P. morganii* (4). Variation on the theme (recognition of new or less common PC-containing microorganisms) could be accomplished by variation in those binding site residues interacting with carrier. Alternatively, the use of M511/M167 (VK-24) L chains could permit accommodation of infrequent PC antigens. What is interesting about T15 and M603 anti-PC antibodies is that variation occurs principally in carrier recognition. Functional variation may also be accomplished by somatic mutations in CDR, e.g., CDR 2 of M603 and W3207.

We are currently testing these ideas in a number of ways. Heterologous recombination experiments among M603, T15, and M511 HP of known sequence should allow us to determine not only the extent to which combinatorial association between families and among members of a family contributes to diversity, but also to assess the role of L chains in fine specificity and to assign a role for DH. We are also examining the binding of HP to PC antigens from a variety of natural sources. This should shed light on the importance of a fixed vs. a variable site and hopefully allow us to determine if DH length or sequence or various somatic mutations in V play significant roles in hapten or carrier recognition.

[3] S. Hudak and J. L. Claflin. Manuscript submitted for publication.

Summary

The present investigation extends our immunochemical characterization of binding site heterogeneity among a large series of monoclonal anti-phosphocholine (PC) antibodies. Hybridoma proteins (HP) from eight genetically distinct strains are included in this study, yet no strain specific characteristics were observed. These HP, as previously shown (5), are divided into three well-defined families based on public or family-specific Id and L chain isotypes characteristic of three PC-binding myeloma proteins: T15, M603, and M511. All antibodies exhibited some degree of inter- or intra-family heterogeneity, or both. Some of this intra-family diversity was reflected by differential reactivity for PC when attached to three different carriers. In spite of this, the specificity profiles for hapten analogues of PC, as measured by hapten inhibition of binding, were the same for all members of the T15 family. Altering the carrier had no effect, thus suggesting that the binding site pocket for PC is essentially preserved, whereas that for carrier is variable. Similar conclusions were reached for most of the M603 HP, although the binding site is different from the T15 HP. The M511 HP stand in sharp contrast to the HP in the other two families because their binding sites exhibit extensive variability. The independence in reactivity for PC and PC plus carrier offers a rational explanation for idiotypic and/or structural heterogeneity within a family. More importantly it suggests interesting strategies for diversification within one group of antibodies.

The authors are indebted to B. Barnes and E. Williams for typing this manuscript, and to Cheryl Rasch for her photographic assistance. The authors thank Dr. Wesley Dunnick for the many fruitful discussions and S. Rudikoff for C3 protein.

Received for publication 3 August 1981.

References

1. Claflin, J. L. 1976. Uniformity in the clonal repertoire for the immune response to phosphorylcholine in mice. *Eur. J. Immunol.* **6**:669.
2. 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.
3. 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.
4. Williams, K., and J. L. Claflin. 1980. Clonotypes of anti-phosphocholine antibodies induced with *Proteus morganii* (Potter). *J. Immunol.* **125**:559.
5. Claflin, J. L., S. Hudak, and A. Maddalena. 1981. Anti-phosphocholine hybridoma antibodies. I. Direct evidence for three distinct families in the murine response. *J. Exp. Med.* **153**:352.
6. Gearhart, P. J., N. D. Johnson, R. Douglas, and L. Hood. 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. *Nature (Lond.)*. **291**: 29.
7. Rudikoff, S., and J. L. Claflin. 1976. Expression of equivalent clonotypes in BALB/c and A/J mice after immunization with phosphorylcholine. *J. Exp. Med.* **144**:1294.
8. Claflin, J. L., and S. Rudikoff. 1979. Structural evidence for a polymorphic or allelic form of the heavy chain variable region. *J. Immunol.* **122**:1402.
9. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1977. The monoclonal anti-phosphoryl-

choline antibody response in several murine strains: genetic implications of a diverse repertoire. *J. Exp. Med.* **145:**876.

10. Pease, L. R., and J. L. Claflin. 1981. Clonal regulation in the response to phosphocholine. II. Heterogeneity among T15 idiotype positive antibodies in inbred and wild mice. *Eur. J. Immunol.* In press.
11. Shulman, M., C. D. Wilde, and G. Köhler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature (Lond.).* **276:**269.
12. Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* **123:**1548.
13. 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.
14. Young, N. M., and M. Leon. 1977. The binding of analogs of phosphorylcholine by the murine myeloma protein McPC603, MOPC167 and S107. *Immunochemistry.* **14:**757.
15. Liu, T.-Y, and E. C. Gotschlich. 1963. The chemical composition of pneumococcal C-polysaccharide. *J. Biol. Chem.* **238:**1928.
16. Potter, M. 1977. Antigen-binding myeloma proteins of mice. *Adv. Immunol.* **25:**141.
17. Rudikoff, S., and M. Potter. 1980. Allelic forms of the immunoglobulin heavy chain variable region. *J. Immunol.* **124:**2089.
18. Lieberman, R., S. Rudikoff, W. Humphrey, and M. Potter. 1981. Allelic forms of anti-phosphorylcholine antibodies. *J. Immunol.* **126:**172.
19. Maddalena, A., and J. L. Claflin. 1981. Idiotypic comparison of antiphosphocholine, T15 Id positive hybridoma and serum antibodies. *J. Supramol. Struct. Cell. Biochem.* **S5:**38.
20. Potter, M., and R. Lieberman. 1970. Common antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphorylcholine. *J. Exp. Med.* **132:**737.
21. Andres, C. M., N. M. Young, and J. L. Claflin. 1981. Characterization of the M511 family of mouse anti-phosphocholine hybridoma proteins. *J. Supramol. Struct. Cell. Biochem.* **S5:**36.
22. Bennet, L. G., and C. P. J. Glaudemans. 1979. Contributions by ionic and steric features of ligands to their binding with phosphorylcholine-specific immunoglobulin IgA H-8 as determined by fluorescence spectroscopy. *Biochemistry.* **18:**3337.
23. Barstad, P., J. Hubert, M. Hunkapiller, A. Goetze, J. Schilling, B. Black, B. Eaton, J. Richards, M. Weigert, and L. Hood. 1978. Immunoglobulins with hapten-binding activity: structure-function correlations and genetic implications. *Eur. J. Immunol.* **8:**497.
24. Leon, M., and N. M. Young. 1971. Specificity for phosphorylcholine of six murine myeloma proteins reactive with Pneumococcus C polysaccharide and β-lipoprotein. *Biochemistry.* **10:** 1424.
25. Imanishi, T., and O. Mäkelä. 1973. Strain differences in the specificity of mouse anti-hapten antibodies. *Eur. J. Immunol.* **3:**323.
26. Reth, M., G. J. Hammerling, and K. Rajewsky. 1978. Analysis of the repertoire of anti-NP antibodies in C57BL/6 mice by cell fusion. I. Characterization of antibody families in the primary and hyperimmune response. *Eur. J. Immunol.* **8:**393.
27. Pierres, M., S.-T. Ju, C. Waltenbaugh, M. E. Dorf, B. Benacerraf, and R. N. Germain. 1979. Fine specificity of antibodies to poly (Glu^{60} $Ala^{30}Tyr^{10}$) produced by hybrid cell lines. *Proc. Natl. Acad. Sci. U. S. A.* **76:**2425.
28. Chesebro, B., and H. Metzger. 1972. Affinity labeling of a phosphorylcholine-binding mouse myeloma protein. *Biochemistry.* **11:**766.
29. Jennings, H. J., C. Lugowski, and N. M. Young. 1980. Structure of the complex polysaccharide C-substance from *Streptococcus pneumoniae* type I. *Biochemistry.* **19:**4712.
30. Padlan, E. A., D. R. Davies, S. Rudikoff, and M. Potter. 1976. Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. *Immunochemistry.* **13:**945.

31. Potter, M., S. Rudikoff, E. A. Padlan, and M. Vrana. 1977. The covalent structure of the antigen binding site: antigen binding myeloma proteins of the BALB/c mouse. *In* Antibodies in Human Diagnosis and Therapy. E. Haber and R. M. Krause, editors. Raven Press, New York.
32. 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.
33. Rudikoff, S., and M. Potter. 1978. K chain variable region from M167, a phosphorylcholine binding myeloma protein. *Biochemistry.* **17:**2703.
34. Appella, E. 1980. Amino acid sequence of the light chain variable region of M511, a phosphorylcholine-binding murine myeloma protein. *Mol. Immunol.* **17:**711.
35. Crews, S., J. Griffin, H. Huang, K. Calame, and L. Hood. 1981. A single V_H gene segment encodes the immune response to phosphorylcholine: somatic mutation is correlated with the class of the antibody. *Cell.* **25:**59.
36. Lieberman, R., M. Potter, E. B. Mushinski, J. R. W. Humphrey, and S. Rudikoff. 1974. Genetics of a new IgV_H (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J. Exp. Med.* **139:**983.