STRUCTURAL, FUNCTIONAL, AND IDIOTYPIC CHARACTERISTICS OF A PHOSPHORYLCHOLINE-BINDING IgA MYELOMA PROTEIN OF C57BL/ka ALLOTYPE*

By J. LATHAM CLAFLIN, STUART RUDIKOFF, MICHAEL POTTER, AND JOSEPH M. DAVIE

(From the Departments of Pathology and Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110 and The National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

During the past several years groups of myeloma proteins that bind specific haptens have been described (1–15) and used as model immunoglobulins for studying structure-function relationships. However, the relatively low affinity expressed by these proteins for their ligands raised the possibility that such interactions represented only weak cross-reactions, and that the myeloma proteins may not, in fact, have been derived from precursor cells which could be stimulated by these ligands. Recent evidence, however, has clearly shown that such reservations are not warranted. Thus, IgM antibodies raised in mice to phosphorylcholine (PC)[1] (16–20), $\alpha(1 \rightarrow 3)$dextrans (21), fructosans, and galactans (M. Potter, R. Lieberman, and B. Mushinski, unpublished observations) possess idiotypic determinants similar to those present on IgA myeloma proteins with the corresponding specificity, which suggests that a close structural similarity exists between the myeloma proteins and antibody molecules. In the PC system, a remarkable uniformity of structural and functional characteristics has been described on members of a group of PC-binding myeloma proteins (S63-T15 group). This group shares idiotypic determinants, the same amino-terminal sequence of heavy and light chains (6), and indistinguishable binding characteristics for a number of choline compounds (4). Two distinct idiotypic determinants have been described, one of which is located in the binding site (22) and the other in a nonbinding site region (3). Of particular interest is the idiotypic determinant located in the binding site (22) since it is associated with anti-PC antibody produced in all inbred strains of mice tested, regardless of allotype (19). This correlates with the finding that the binding specificity of anti-PC antibody from each of these mouse strains is indistinguishable from that of T15 (23). This finding suggests that a particular PC-binding site, but not necessarily a nonbinding site region, may be conserved in mice (19, 23). Thus, any PC-binding myeloma protein with a T15-binding site, but having a non-BALB/c allotype, becomes relevant as a possible prototype for anti-PC antibody of that particular allotype group.

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[1] *Abbreviations used in this paper:* AC, acetylcholine; C, choline; GPC, glycerophosphorylcholine; PC, phosphorylcholine; PnC, pneumococcal C polysaccharide; PTH, phenylthiohydantoins.

We report here a PC-binding myeloma protein whose heavy chain carries the allotypic determinants of the a^2 group (e.g. C57BL/6). The tumor was induced in a congenic strain of BALB/c mice (strain CB-20) which carries the C57BL/ka·IgC_H complex locus (24). This strain was obtained by introgressively backcrossing the IgC_H complex locus of C57BL/ka (denoted by the unassigned 2 allotypic determinant) to BALB/c for 20 consecutive generations and then making the 20th generation homozygous for the C57BL/ka IgC_H 2 determinant by brother-sister mating. Plasmacytoma CBPC 2, induced in one of these CB-20 mice, produces an IgA protein which carries the C57BL/ka allotype and shows certain structural and functional characteristics, particularly in the combining region, which are similar to those of the S63-T15 group. In addition, this myeloma protein possesses the site-associated idiotypic determinant, but, like the induced C57BL/6 anti-PC antibody, it does not carry the nonbinding site determinant.

Materials and Methods

Source of Tumors. The origins of the plasmacytomas TEPC 15, HOPC 8, MOPC 167, McPC 603, and MOPC 511 that produce the PC-binding myeloma proteins T15, H8, M167, M603, and M511 (all IgA, κ) have been described in detail elsewhere (3). The plasmacytoma CBPC 2 was induced in a CB-20 mouse that had been injected intraperitoneally with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane; Aldrich Chem. Co., Inc., Milwaukee, Wis.) and then 40 days later with Abelson Virus (MLV-A) (25). This plasmacytoma produced an IgA (α, κ) myeloma protein that precipitated the PC-containing LB-4 antigen of *Lactobacillus acidophilus*.

Purification of Myeloma Proteins. The PC-binding myeloma proteins were purified by affinity chromatography from a PC-Sepharose immunoadsorbent column (26). The isolated proteins were shown to be pure by immunodiffusion with class- and type-specific antisera prepared in rabbits.

Amino-terminal Sequence Determination. Heavy and light chains were separated on Sephadex G100 columns in 6 M urea, 1 M acetic acid after partial reduction and alkylation as previously described (27). Amino-terminal sequence analysis was performed on a Beckman 890 C sequencer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using the standard dimethylallylamine program. Thiazolinone derivatives obtained after each degradation cycle were converted to phenylthiohydantoins (PTH) as previously described (13). PTH amino acids were identified by gas chromatography on SP400 columns (28) and/or by amino acid analysis after hydrolysis in hydriodic acid (29).

Measurement of Binding Specificity. Pneumococcal C polysaccharide (PnC) was isolated from *Diplococcus pneumoniae* strain R36A according to the procedure of Liu and Gotschlich (30). Tyramine was introduced into PnC (31), and the tyraminyl carbohydrate was iodinated with ^{125}I by the chloramine T method (32). Inhibition of precipitation (4) between [^{125}I]PnC and the myeloma proteins by PC and three of its analogues, glycerophosphorylcholine (GPC), acetylcholine (AC), and choline (C), was used to determine the binding specificity of this protein. All reactions were carried out at equivalence in 0.15 M NaCl buffered with 0.005 M phosphate (PBS). 0.2 ml of a serum dilution containing the myeloma protein was incubated with 0.1 ml of various concentrations of the choline analogues. After 30 min at 24°C, 0.1 ml of PBS containing 100 μg (34,000 cpm) of [^{125}I]PnC was added. The reaction mixture was incubated for 16–18 h at 4°C and the precipitate washed four times in PBS; the amount of radioactive precipitate was determined in a gamma counter.

Isoelectric Focusing in Polyacrylamide Gels. Purified myeloma proteins were fully reduced with 0.2 M 2-mercaptoethanol in 7 M guanidine, 0.5 M Tris-HCl, pH 8.6 (2 h, 22°C) and alkylated with 0.4 M iodoacetamide (45 min, 4°C) (33). A portion of each sample was dialyzed against 6 M urea and stored at −20°C. The major portion of the sample was separated into heavy and light chains by Sephadex G100 chromatography in 1 M propionic acid, 4.5 M urea. Isolated heavy and light chains were dialyzed against 6 M urea and stored at −20°C.

Isoelectric focusing in thin layer polyacrylamide gel slabs was performed at 4°C in the apparatus described by Reid and Bieleski (34). The gel was composed of 5% acrylamide (Bio Rad Laboratories,

Richmond, Calif.), 2% ampholytes (Ampholines, LKB Instruments, Inc., Rockville, Md.), pH range 5.0 to 9.5, and 6 M urea (Ultrapure grade; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). Phosphoric acid, 0.5% was used for the anode solution (upper trough) and ethanolamine, 0.8%, for the cathode solution (lower trough). Samples were focused for 12–14 h at a constant current of 2.5 mA and then for an additional hour at 800–900 V. Gels were fixed in a solution of 5% trichloroacetic acid and 5% sulfosalicylic acid for 4 h, washed repeatedly in 40% ethanol, and air dried. Protein bands were detected by staining the dried gels for 4–12 h with 0.025% Coomassie brilliant blue dissolved in 40% ethanol.

Idiotypic Antisera. The preparation and characterization of two idiotypic antisera which react with T15 and H8 are described in detail elsewhere (19, 20, 22). One antiserum, prepared in rabbits to H8, was passed over an H8-Sepharose immunoadsorbent and antibodies with specificity for the binding site were isolated by elution with PC. This antibody preparation, designated R2 anti-$H8_s$, was idiotypically specific for H8 and T15, and this reaction was greater than 98% hapten inhibitable (22). Another antiserum to H8 was prepared in A/J mice (19, 20) and made idiotypically specific by adsorption to Sepharose coupled with M460 (9 mg/ml) in BALB/c serum. Though this antiserum was also idiotypically specific, it was directed primarily to determinant(s) not directly associated with the combining site (22).

Detection of Idiotypic Determinants. The hemagglutination-inhibition (HI) test was performed as previously described (19, 20). Purified myeloma proteins were tested for their ability to inhibit the agglutination of T15-coated sheep erythrocytes (SRBC) (35) by a limiting dilution of idiotypic antiserum. The inhibition endpoint was the highest dilution of inhibitor which gave no visible hemagglutination.

Quantitative measurements of an idiotypic specificity were determined by solid phase radioimmunoassay (TBA) which has been previously described (22). Micro-test tubes (Beckman Instruments, Inc., Fullerton, Calif.) coated with idiotypic antibodies were incubated with ~0.5 ng/ml of [^{125}I]H8 (6,000 cpm) (32) in the presence of various amounts of different myeloma proteins. The bound radioactivity was measured in a gamma counter and the percent inhibition of specific binding was determined. Specific and nonspecific binding to antibody-coated tubes was 33% and 4%, respectively, of added cpm.

Results

Allotypic Specificity of CBPC 2. BALB/c IgA immunoglobulins carry the $A^{12, 13, 14}$ determinants (24), whereas those of C57BL/ka carry the A^{15} determinant. As shown by the immunodiffusion pattern in Fig. 1, CBPC 2 protein was A^{15} which indicates that the heavy chain constant regions of this protein were of C57BL/ka origin. The origin of the light chains is undetermined since K chain allotypes are not known in the mouse.

Amino-terminal Sequence Analysis. The results of partial amino acid sequences of both the light and heavy chains of CBPC 2 are shown in Table I and compared with those of H8, T15, and M603. As indicated previously, H8 and T15 have identical sequences through the first hypervariable region of both their heavy and light chains (6) and appear on the basis of these and other data (3, 20, 22) to be identical molecules. The heavy chain of M603 is identical to T15 through the first hypervariable region but its light chain differs by 18 out of 41 residues, including eight in the hypervariable region (27). CBPC 2 shows a striking sequence similarity to T15. No differences are seen in the light chains through position 36 and only two changes occur in the first 36 residues of the heavy chain, both of which are outside the hypervariable region.

Isoelectric Focusing Pattern of Light Chains. An additional measure of structural similarity among proteins is the isoelectric point (pI). Therefore, the heavy and light chains of T15, CBPC 2, and other PC-binding proteins were analyzed by isoelectric focusing. Preliminary experiments indicated that clear

distinctions among myeloma proteins could be made if fully reduced, rather than mildly reduced, proteins were analyzed. Light chains typically gave from one to four bands after mild reduction depending on the protein, but yielded three to four bands after complete reduction (36). The isoelectric spectra of light and heavy chains of CBPC 2, T15, H8, M603, M511, M167, and of M460, a DNP-binding IgA myeloma protein, are shown in Fig. 2. The light chains focused in restricted regions over a broad range in pH, 5.5 to 8.0. As can be clearly seen, T15 and H8 light chains, as anticipated, but also CBPC 2 light chains, focus with identical pI's. In addition, M511 and M167 light chains, which differ by only a single amino acid in their amino-terminal regions (37), focus similarly. That isoelectric focusing can readily reveal compositional differences as well as similarities is demonstrated by the light chain patterns obtained with T15, M603, M167, and M460 proteins, which belong to entirely different subgroups (6). Thus, the data indicate that other portions of the CBPC 2 light chain, in addition to the amino-terminal regions, are similar to those of T15.

Unlike γ-chains (38), the α-chains, after either full or mild reduction, entered the gel poorly and, even then, were not readily resolved into distinct bands. This may relate to the higher carbohydrate level in IgA and the lability of sialic acid residues.

Binding Specificity of CBPC 2. Measurement of the relative binding specificity of the PC-binding myeloma proteins was used as a criterion for comparison of their combining sites. As shown by other laboratories as well as our own (4, 18), each of the proteins, T15 (H8), M603, M167, and M511, binds PC, but their

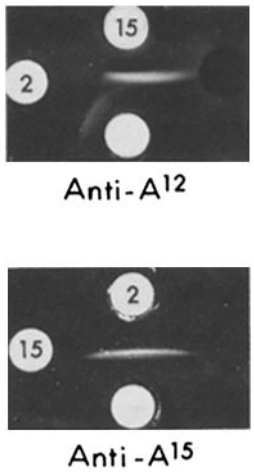


FIG. 1. Identification of allotypic determinants on CBPC 2. *Upper:* Ouchterlony plate showing interaction of AL/N anti-BALB/c IgA myeloma protein X44 (anti-A^{12}) with CBPC 2 (2) and T15 (15). The precipitin line formed with the T15 is partially inhibited by the CBPC 2 myeloma protein indicating some shared allotypic specificities between C57BL and BALB/c IgA myeloma proteins. The strong precipitin line with T15 and not CBPC 2 indicates the presence of BALB/c IgA allotype A^{12} on T15 and its absence on CBPC 2. *Lower:* BALB/c anti-IgA myeloma protein M320 (anti-A^{15}). M320 was produced by a plasmacytoma induced in a backcross-6 mouse during development of the CB-20 congenic strain and possesses the C57BL/ka IgC_H alpha chain. The antiserum shows the presence of the C57BL IgA allotype on CBPC 2 and its absence on T15.

TABLE I

Amino-terminal Sequences of Myeloma

V_L

	5	10	15	20
T15 (H8)*	Asp Ileu Val Met Thr Gln Ser Pro Thr Phe Leu Ala Val Thr Ala Ser Lys Lys Val Thr			
CBPC 2	———			
M603*	———	Ser Ser	—Ser—Ser	—Gly Glu Arg——

V_H

	5	10	15	20
T15 (H8)*	Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu			
CBPC 2	———	———	Ser — Arg—	———
M603*	———	———	———	———

* Sequences determined by Barstad et al. (6) and by Rudikoff and Potter (27).

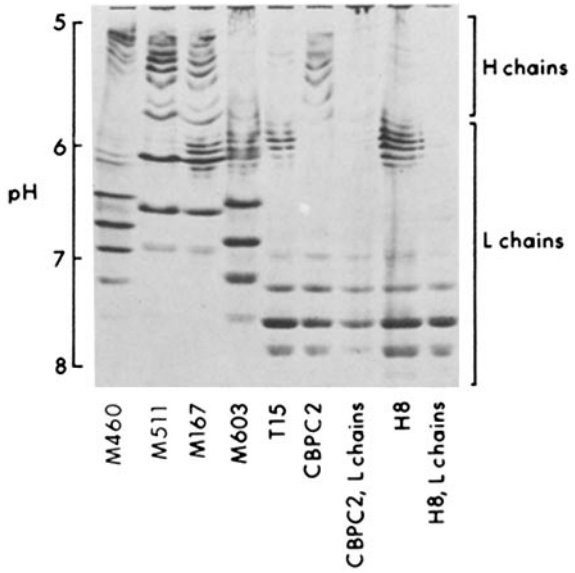
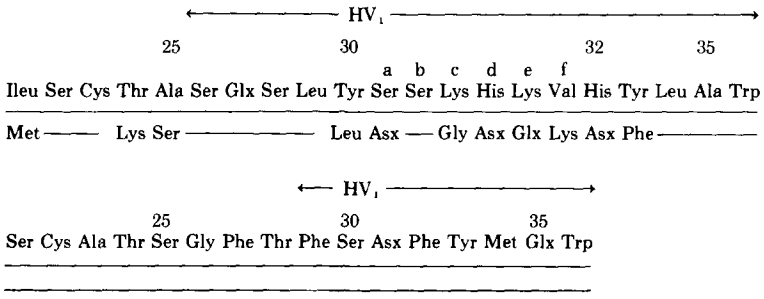


FIG. 2. Isoelectric spectra of heavy and light chains of PC-binding myeloma proteins. Reduced and alkylated proteins and isolated light chains were focused in a thin layer polyacrylamide gel in pH 5 to 9.5 ampholytes. With the exception of the major dark band at pH 5.9 in M167, the multiple banding appearing at pH 6.0 ± 0.2 in M167, M603, T15, and H8 is due to an unidentified, low molecular weight contaminant.

combining sites display readily distinguishable fine specificity differences. Comparative analysis of binding characteristics for CBPC 2 and 3 of the 4 different PC-binding proteins are shown in Fig. 3 and Table II. It is readily apparent that the combining site of CBPC 2 is markedly similar to that of T15. Though differing slightly in relative affinity for each of the choline analogues (a fact which is most likely due to a variation in immunoglobulin concentration [4]), they each possessed virtually the same relative specificity for the choline analogues.

Proteins that Bind Phosphorylcholine



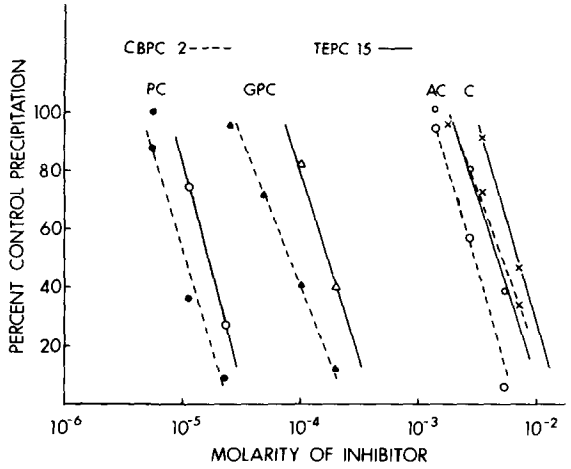


FIG. 3. Binding specificity of CBPC 2. Inhibition of precipitation of pneumococcal C polysaccharide by phosphorylcholine (PC), glycerophosphorylcholine (GPC), acetylcholine (AC), and choline (C).

TABLE II

Binding Specificity of Myeloma Proteins that Bind Phosphorylcholine

Tumor proteins	I_{50}*				Relative specificity‡		
	PC	GPC	AC	C	GPC/PC	AC/PC	C/PC
TEPC 15	0.016	0.17	4.8	7.0	10.3(11)§	296(380)	427(550)
CBPC 2	0.010	0.082	3.0	5.2	8.2	300	520
MOPC 167	0.032	0.041	0.30	0.35	1.3(0.8)	9.6(10)	11(10)
McPC 603	0.021	0.183	18.2	19.5	8.9(10)	885(1,000)	950(870)

* I_{50} = m molar concentration of hapten which gives 50% inhibition of binding of [^{125}I]PnC to myeloma protein.

‡ Relative specificity = (molarity of GPC, AC or C at I_{50})/(molarity of PC at I_{50}).

§ Numbers in parentheses are values reported by Leon and Young (4).

Idiotypic Determinants on CBPC 2 Protein. Work from these laboratories has shown that H8 and T15 share two different idiotypic determinants that are not found on M511, M167, or M603. One of these determinants, (T15), detected by an antiserum produced in A or AL mice, is not modified by PC binding and therefore appears to lie outside the combining site (19, 22). In contrast, the other determinant(s), ($H8_s$), detected by antibodies isolated from a rabbit anti-H8 serum, is in the binding site since it is completely inhibited by hapten (22). As shown by the hemagglutination-inhibition reaction (Table III) and by solid phase radioimmunoassay (Fig. 4) only the $H8_s$ determinant(s) was present on CBPC 2. No binding of CBPC 2 by A anti-H8 occurred, even when CBPC 2 was used at 100,000 times the effective concentration of T15. In contrast, at a concentration of 20 ng/ml, CBPC 2 inhibited the binding of R2 anti-$H8_s$ to T15-SRBC to a

TABLE III

Idiotypic Specificities of PC-Binding Myeloma Proteins

Immunoglobulin	HI titer*	
	R2 anti-$H8_s$	A/J anti-H8
	mg/ml	*mg/ml*
BALB/c NMG	>2	>2
CBPC 2	0.000020	2
HOPC 8	0.000040	0.000080
TEPC 15	0.000020	0.000020
MOPC 167	>2	>2
MOPC 511	>2	>2
McPC 603	>2	>2

* Mean determined from three separate experiments. Data represent the concentration of protein giving complete inhibition of the reaction between H8-coated sheep erythrocytes and the rabbit (R2 anti-$H8_s$) or mouse (A/J anti-H8) idiotypic antibody.

degree comparable to that obtained with T15 and H8. This is expressed in quantitative terms by the TBA shown in Fig. 4. On a weight basis CBPC 2 and T15 give comparable inhibition of binding which suggests that the determinants in their combining sites are idiotypically very similar, if not identical.

Discussion

The present studies demonstrate that a PC-binding myeloma protein, CBPC 2, whose heavy chain is of C57BL/ka (a^2) origin, possesses variable region structural and functional characteristics that are similar to those of a group of PC-binding myeloma proteins (S63-T15) of BALB/c (a^1) origin. This conclusion is based on a variety of criteria including antibody binding specificity, amino-terminal heavy and light chain sequences, light chain mobility and binding site antigenic determinants. This protein, however, is distinguished from the myeloma proteins in the S63-T15 group since it carries C57BL/ka, instead of the BALB/c, allotype markers and by the fact that it does not carry the nonbinding site idiotypic determinant, T15.

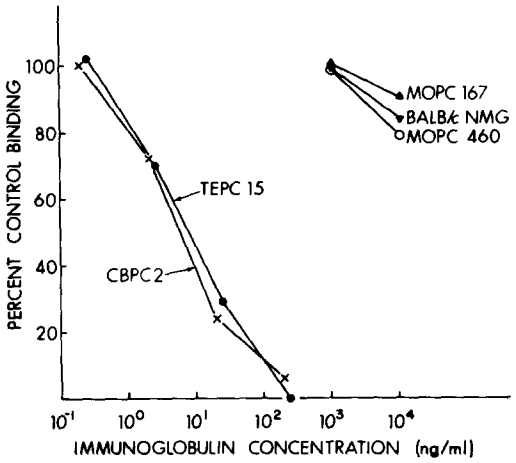


FIG. 4. Idiotypic similarity of $H8_s$ determinant in T15 and CBPC 2. Inhibition of binding of [^{125}I]H8 to anti-$H8_s$-coated tubes.

The possession of the C57BL/ka (C_H) heavy chain allotype suggests that the V_H heavy chain of CBPC 2 is of C57BL/ka origin. This is supported by the fact (20, 39) that the congenic strain CB-20 has other V_H genes of C57BL/ka origin and by the finding that the heavy chain sequence of CBPC 2 contains amino acids in at least two positions (14 and 16) which are not found in any of 29 BALB/c myeloma proteins (see 40). Thus, it seems probable that the heavy chain of CBPC 2 was derived intact from genetic information provided by the C57BL/ka mouse. The origin of the light chain of CBPC 2 cannot be determined at present since no light chain allotypes on mouse kappa chains have been defined. Furthermore, the V_L I_B-peptide marker described by Edelman and Gottlieb (41) is not found in either BALB/c or C57BL/ka mice. While there is no a priori reason why the same light chain may not be expressed in more than one strain of mice, it is equally likely that the CBPC 2 light chain is derived from the BALB/c genome since the CB-20 strain was selected for allotype, and heavy and light chain genes are not linked.

The observation that CBPC 2 possesses the $H8_s$ determinant and the same binding specificity as T15 supports the idea that these two proteins have the same structure in the combining site. However, CBPC 2 differs structurally from T15 in some portion of the variable region, since it lacks the nonbinding site T15 idiotype. The location of this antigenic determinant will have to await further experimental analysis. It is pertinent to note, however, that the hypervariable loops of one of the PC-binding proteins, M603, are gathered together to form a cleft which is much more extensive than that occupied with PC binding (42). Secondly, the second hypervariable region of the light chain of M603 (42) and also of the human myeloma protein, New, (43) is removed from the binding site and is accessible from the outside. If this region in T15 varied from that in CBPC 2, it would be a candidate for the T15 idiotype. However, the possibility that idiotype markers exist outside hypervariable regions still appears possible. In this regard, positions 14 and 16 of the V_H in the M603 are on the exterior surface of the

molecule and the interchanges found at these positions in CBPC 2 could conceivably be nonbinding site determinants.

Recent studies (3–15) of groups of myeloma proteins have indicated a striking correlation between binding specificity, cross-idiotypic determinants and amino-terminal sequences of either the heavy chain and/or light chain. Proteins that bind $\beta(1 \rightarrow 6)$-D-galactan are especially intriguing since they show similar sequences in both their heavy and light chains (10, 12, 13). Perhaps the most pertinent to the present study, since they arose in genetically different individuals, are the human monoclonal cold agglutinins and antigamma globulins studied by Kunkel, Capra, and their colleagues (44, 45). Though no data on heavy chain structure have been presented, amino acid sequence analysis of the light chains from 6 of 10 cold agglutinins showed minimal differences among them (46). Likewise, antigamma globulins selected for similarity in shared idiotypic determinants exhibit remarkable structural similarities in either their light or their heavy chains (47). However, no two proteins appear to be completely identical in either structure or in individual antigenic specificity.

Thus, CBPC 2 and those in the S63-T15 group of PC-binding myeloma proteins, at present, present a unique finding among myeloma proteins derived from the mouse or the human. Though structural differences exist between CBPC 2 and S63-T15, these relate to either allotypic characteristics in the C_H region or to a variable region(s) difference outside the combining site. No detectable differences have been found in the combining site, which indicates that this portion of the molecule has been preserved in two mouse strains that exhibit considerable differences in their genetic make-up. In this regard, it has been shown previously that both C57BL/6 and BALB/c, as well as all mouse strains tested, produce anti-PC antibodies which show the same specificity (23) and binding site antigenic determinants (19) as those of T15 and CBPC 2. If indeed sequence analysis verifies the conservation of this binding site in *Mus musculus*, this family of immunoglobulins will offer an excellent opportunity to study variable region diversity as well as permit analysis of structure-function-idiotype relationships.

Summary

An IgA phosphorylcholine (PC)-binding myeloma protein with IgC_H allotypic determinants different from those of BALB/c mice is characterized. The myeloma, CBPC 2, was induced in the CB-20 strain of mice which is congenic to BALB/c but differs from it by carrying the A^{15} allotypic determinant of C57BL/ka mice. Sequence analysis of the CBPC 2 light chain through the first hypervariable region, as well as isoelectric point analysis, show that this chain is indistinguishable from that of T15, a PC-binding myeloma protein of BALB/c origin. The heavy chains of CBPC 2 and T15 differ by only two amino acids (positions 14 and 16) through the first hypervariable region. As measured by inhibition of precipitation, both CBPC 2 and T15 have the same specificity for PC, glycerophosphorylcholine, acetylcholine, and choline. In addition, CBPC 2 possesses the binding site-associated idiotypic determinant which is present on T15. However, like normal or induced C57BL/6 anti-PC antibody, it does not possess the nonbinding site idiotypic determinant.

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