SITE-DIRECTED MUTAGENESIS OF L96 OF PRIMARY AND SECONDARY IMMUNE RESPONSE ANTIBODIES AGAINST PC-KLH

by

Arun Bhat

A DISSERTATION

Presented to the Department of Molecular Microbiology and Immunology
and
the Oregon Health Sciences University School of Medicine
in partial fulfillment of the requirements for the degree of

Master of Science

December, 1994

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like express my gratitude to Dr. Marvin Rittenberg and Mary Stenzel-Poore who together as one taught me the skills required of a good scientist. As with anything in life, one can only learn so much before moving on.

I would also like to specially thank all the people in the laboratory who helped me in my endeavors. Tammy Martin for helping me with the experimental work, McKay Nutt, Ching Chen, Susan Stevens, Gregory Wiens and Christine Kowalczyk who showed me the value of a true friendship.

Finally, I would like to thank my family: my father Dr. Bal Krishen Bhat for whom the quote, friend, philosopher, and guide, truly fits as he has been all these and much more to me. My mother Mrs. Sarla Bhat who has always been there, even when things weren't going my way. And to my "little" sister Anupama Bhat who has been a sister and a true friend I can rely on. Lastly, I would like to share a quotation that my father faxed me one day. This quotation really sums up for me what science and life is all about.

> Nothing in this world can take place of persistence. Talent will not; nothing is more common than unsuccessful people with talent. Genius will not; unrewarded genius is almost a proverb. Education will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent. The slogan "press on" has solved and always will solve the problems of the human race.
>
> Calvin Coolidge

ABBREVIATIONS

BSA	bovine serum albumin
CDR	complementarity determining region
ELISA	enzyme-linked immunosorbent assay
GPC	L-α-glycerophosphocholine
Ig	immunoglobulin
J	joining region
KLH	keyhole limpet hemocyanin
κ	kappa light chain
λ	lambda light chain
mab	monoclonal antibody
NPDBP	p-nitrophenyl-3, 3-dimethylbutylphosphate
NPPC	p-nitrophenyl phosphocholine
NIP	4-hydroxy-3-iodo-5-nitrophenylacetic acid
NPSo	nitrophenyl-soman
OVA	ovalbumin
OX	2-phenyl-oxazolone
PC	phosphocholine
VH	heavy chain variable region
VL	light chain variable region

ABSTRACT

$T15^+$ antibodies are the predominant antibodies elicited against phosphocholine (PC)-protein conjugates during the primary immune response. These antibodies contain a leucine at the light chain (L) junctional residue, position 96 in the third complementarity determining region. Whereas, in the memory response a variety of antibodies are elicited that predominantly contain an aromatic amino acid at this position. We examined the importance of position L96 in the prototypic primary immune response antibody T15 and a prototypic memory antibody M3C.65. Using site-directed mutagenesis we have investigated the role of this residue in conferring the fine-specificity binding patterns peculiar to T15 and M3C.65. We replaced the leucine L96 in T15 by an aromatic amino acid, either tyrosine, tryptophan, or phenylalanine, whereas the tryptophan L96 in M3C.65 was replaced by a leucine. Expression of all replacement mutants in T15 at the leucine 96 position revealed that they were severely impaired in binding to PC analogs. On the other hand, the mutant at tryptophan 96 in M3C.65 retained significant binding activity. We also demonstrated that these residues in T15 and M3C.65 do not directly confer the binding specificity in these antibodies since there was no evidence by ELISA that introducing an aromatic amino acid at L96 in T15 was capable of altering specificity whereas M3C.65 likewise did not show any shift in specificity towards a T15-like binding profile. Molecular modeling studies suggest steric hindrance to be a possible explanation for the abolishment of binding when an aromatic amino acid is substituted for leucine at position 96 in the T15 VL region. Thus it appears that this residue is a critical feature in antigen binding of T15 antibodies although it does not represent a residue that solely confers the T15 or memory phenotype as determined by fine specificity binding studies

Literature Review

General features of the anti-phosphocholine (PC) response

Phosphocholine is a simple chemically defined zwitterionic moiety that is naturally abundant in cell walls of certain bacteria (Tomasz 1967; Pèry et al., 1974; Potter 1971). Extensive research has been carried out to study the development of the immune response against this hapten. Upon immunizing BALB/c mice with cell wall extract from *Streptococcus pneumoniae* rough strain R36a, a nearly monoclonal T15 idiotype (T15 id) positive response develops which is protective to the mouse (Sher and Cohn, 1972; Claflin 1976; Briles et al., 1981). These T15id positive antibodies comprise greater than 90% of the primary antibody population (Lieberman et al., 1974) whereas the minor populations represented by the myeloma lines McPC 603 and M167/511 comprise less than 10% (Rudikoff 1983). Upon secondary immunization class switching and somatic mutation is seen in the minor population. The T15id antibodies undergo little if any somatic mutation, suggesting that this antibody may be perfectly suited for binding to PC and that somatic mutation was likely to be harmful (Perlmutter et al., 1984; Crews et al., 1981; Clarke et al., 1983). This hypothesis was tested in our laboratory by a previous graduate student Ching Chen (Chen et al., 1992) who carried out saturation mutagenesis of the CDR2 region of T15 antibody. Chen found as predicted that T15 was highly sensitive to the harmful effects of somatic mutation and that more than 50% of mutations in VH CDR2 generated antibodies with either lower binding affinity than wt or that had lost antigen binding ability completely. The presence of high levels of phosphocholine-binding T15 id positive antibodies in normal serum of virtually every BALB/c mouse examined by Lieberman et al., (1974) suggested that this is a natural antibody which may develop in response to the products of microorganisms in the gastrointestinal and respiratory tracts. A variety of

microorganisms, some in the intestinal tract of mice are known to produce phosphorylcholine, for example it is found in *Streptococcus*, *Morganella*, *Aspergillus*, *Nippostrongylus* and *Ascaris* (Tomasz 1967; Pèry et al., 1974; Potter 1971). It has been proposed that animals contacting PC-carrying microbes in the gut are exposed and primed to the PC moiety (Lieberman et al., 1974). Thus far, the PC moiety has always been found attached to a carbohydrate backbone.

In our laboratory we examined the development of the PC response to PC coupled to KLH via a diazophenyl linkage (Chang et al., 1982, 1984). In this antigen PC is attached to an amino acid (mainly tyrosines and histidines) via a phenyl group (Stenzel-Poore et al., 1988). This phenyl group, becomes important in the secondary immune response to PC-KLH (Chang et al., 1984; Stenzel-Poore et al., 1987, 1988, 1989). Secondary immune response antibodies made against PC-protein conjugates use much more diverse H chain and L chain genes, however, they are not likely to afford any protection to the animal since they cannot bind PC bound to a carbohydrate backbone (Chang et al., 1984; Stenzel-Poore et al., 1987, 1988, 1989).

These memory antibodies differ from the primary antibodies in their fine specificity binding pattern. They recognize PC-protein and nitrophenyl-PC (NPPC) but not PC itself or PC-polysaccharide (Stenzel-Poore et al., 1988), whereas the primary response antibodies recognize PC, choline, α-glycerophosphocholine, NPPC, PC-polysaccharides, and PC-protein (Briles et al., 1981).

Antibody structure

Antibody molecules have multiple functions: via the F_V they can bind to a plethora of antigens whereas via the F_C portion many effector functions are mediated including

complement cascade activation, opsonization, antibody-dependent cell-mediated cytotoxicity (ADCC), and mast cell degranulation (Golub and Green 1991). These functions make antibodies key components of the mammalian immune response. Immunoglobulin G (Ig G) antibodies are tetramers composed of two identical light chains and two identical heavy chains joined by disulfide linkages (see Fig. 1). Each of these chains is composed of an NH_2-terminal variable region (~100 amino acids) and a carboxy-terminal constant region (~100 amino acids in light chains and 300-400 amino acids in heavy chains, depending on the particular class). It is the variable regions of the light and heavy chains that are involved in the formation of the antigen-binding site (Golub and Green 1991).

All antibody domains, whether variable or constant, form compact globular structures with a characteristic fold, termed the immunoglobulin fold by Poljak et al., (1973). Each domain consists of a stable arrangement of hydrogen-bonded, anti-parallel β-strands which form a bilayer structure, stabilized by a disulfide bond between the two layers. In the variable domains, the bilayer structure is formed by nine strands; in the constant domains, the bilayer is formed by seven strands. Bends of different sizes and conformations connect the strands. The predominant secondary structure in antibodies is the anti-parallel β-sheet, with short stretches of α-helix found in some bends (Padlan 1994).

Immunoglobulin light chains are generated from three gene segments, designated variable (V_L), joining (J_L), and constant (C_L) (Brack et al., 1978; Weigert et al., 1978; Max et al., 1979; Sakano et al., 1979). The κ-chain V_L segments encode amino acids 1-95 of the variable region; J_L, amino acids 96-113; and C_L, the entire constant region. Position 96, encoded by the J_L segment, is the last residue in the third light chain complementarity-determining region, thus potentially enabling substitutions at this

position to introduce diversity into the antigen-binding site. Heavy chains are similarly encoded by variable (V_H) (Early et al., 1980; Sakano et al., 1980), joining (J_H) (Rao et al., 1979; Bernard and Gough, 1980; Early et al., 1980; Schilling et al., 1980; Sakano et al., 1980), and constant (C_H) (Honjo and Kataoka, 1978; Yaoita and Honjo, 1980; Rabbitts et al., 1980; Shimizu et al., 1981) gene segments and in addition employ a third variable region segment, D (D_H) (Early et al., 1980; Sakano et al., 1980, 1981; Kurosawa and Tonegawa, 1982). The V_H genes encodes amino acids 1-~95; D_H, a portion of the third complementarity region; J_H, amino acids 100b-113, and C_H the entire constant region.

On the basis of sequence variation, the residues in the variable domains are assigned either to hypervariable (CDR), or to nonhypervariable or framework regions (Wu and Kabat, 1970). The CDRs of the light chain are defined as residues 24-34 (CDR1-L), 50-56 (CDR2-L) and 88-97 (CDR3-L); those of the H chain contain residues 25-31 (CDR1-H), 50-56 (CDR2-H) and 95-101 (CDR3-H). The framework regions in the VL are defined as residues 1-23 (FR1-L), 35-49 (FR2-L), 57-87 (FR3-L) and 98-107 (FR4-L); those in the VH contain residues 1-30 (FR1-H), 36-49 (FR2-H), 66-94 (FR3-H), and 102-112 (FR4-H).

In the crystal structures of F_{ab} fragments, the CDRs of L and H chains are seen as loops mainly situated at the tip of the V region where they form a continuous surface approximately 2800 $Å^2$ in area (Padlan 1994). The conformation of each CDR is primarily determined by the nature and number of amino acids in the segment which varies from 5 to 15 residues in length (Padlan 1994; Golub and Green 1991). The variability in sequence and size seen in the CDRs results in a large variation in the topography of the CDR surface.

The binding site of McPC 603, is a large wedge-shaped cavity which is lined by five of the six CDR loops, namely CDR1-L, CDR3-L, CDR1-H, CDR2-H and CDR3-H (see Fig. 2). PC was found to bind to M603 in this cavity with the choline group buried deep in the interior and the phosphate moiety more towards the exterior of the cavity. The hapten interacts mainly with the H chain residues. Electrostatic interactions, hydrogen-bonding and van der Waals forces all contribute to the interaction. The positively charged trimethylammonium moiety of the hapten is in contact with the carboxyl of Asp L91 and partially neutralized by Glu H35 and Glu H58. The negatively charged phosphate interacts with the positively charged guanidium group of Arg H52 (see Fig. 3). Sequence analysis of a number of PC binding antibodies such as T15, M167 and M511, suggested that the 3D structure observed in M603 and the mode of binding to the hapten is representative of those antibodies also (Padlan et al., 1985, 1976).

FIGURE 1. Schematic representation of an IgG molecule and active fragments that can be derived from it. These were originally defined by differential proteolytic cleavage, but are now commonly produced by genetic engineering. The divalent $F(ab')_2$ fragment (large box, heavy outline) includes the hinge region and is produced by cleavage with pepsin. Papain digestion yields the F_c fragment (bounded by broken outlines; carbohydrate in C_H2 indicated between domains) and two identical F_{ab} fragments (for clarity, one is shown against a shaded brackground). Under reducing conditions the F_{ab} is resolved into the light chain and the F_d (in box). The light- and heavy-chain variable regions comprise the F_v, the monovalent antigen-binding site; it can be obtained, with difficulty, by acid hydrolysis. (Taken from Wright et al., 1992).

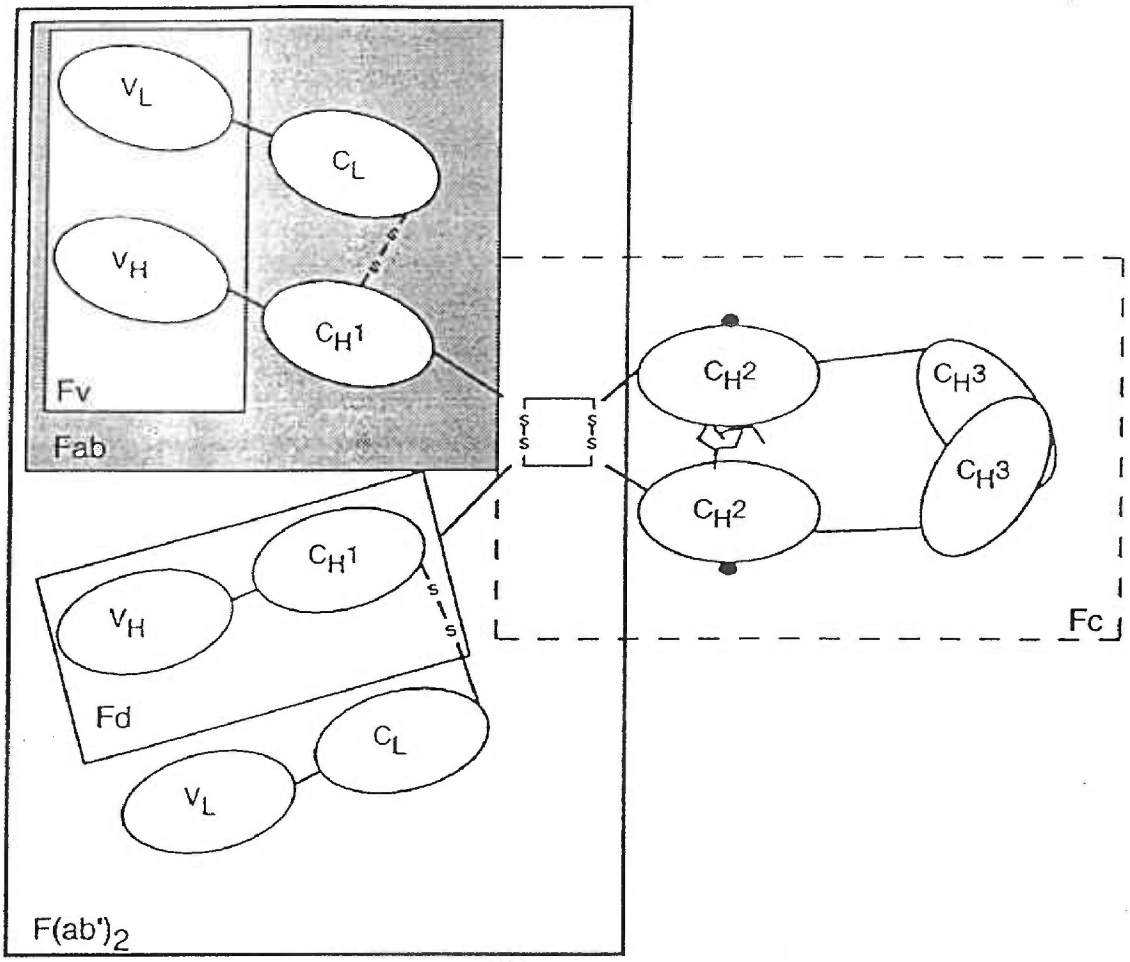

V_L
C_L
V_H
C_H1
Fv
Fab
C_H2
C_H3
C_H3
C_H2
Fc
C_H1
V_H
Fd
C_L
V_L
$F(ab')_2$

FIGURE 2. Three-dimensional structure of the McPC 603 F_{ab}. Complementarity-determining regions are indicated by solid circles. (Taken from Davies et al., 1975).

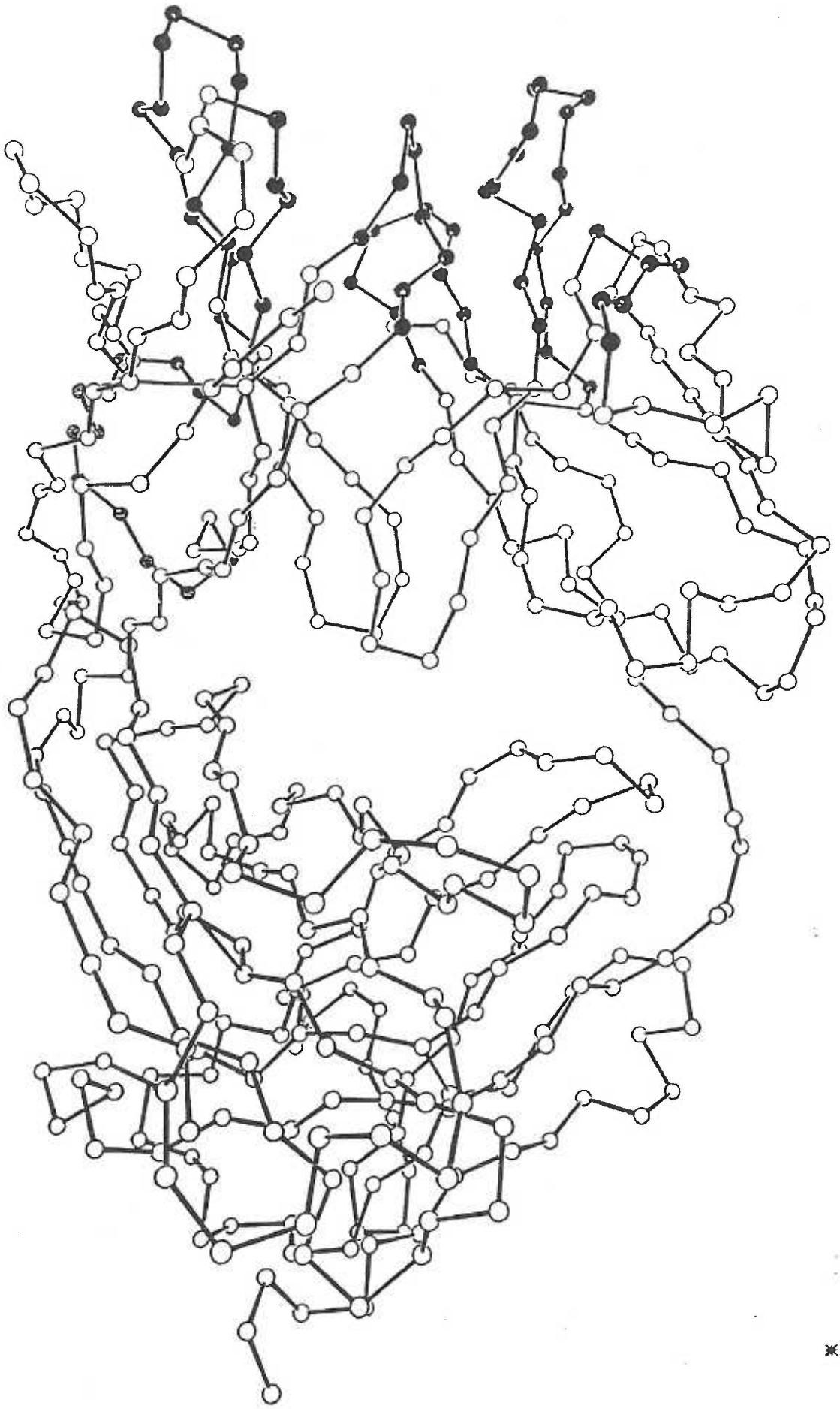
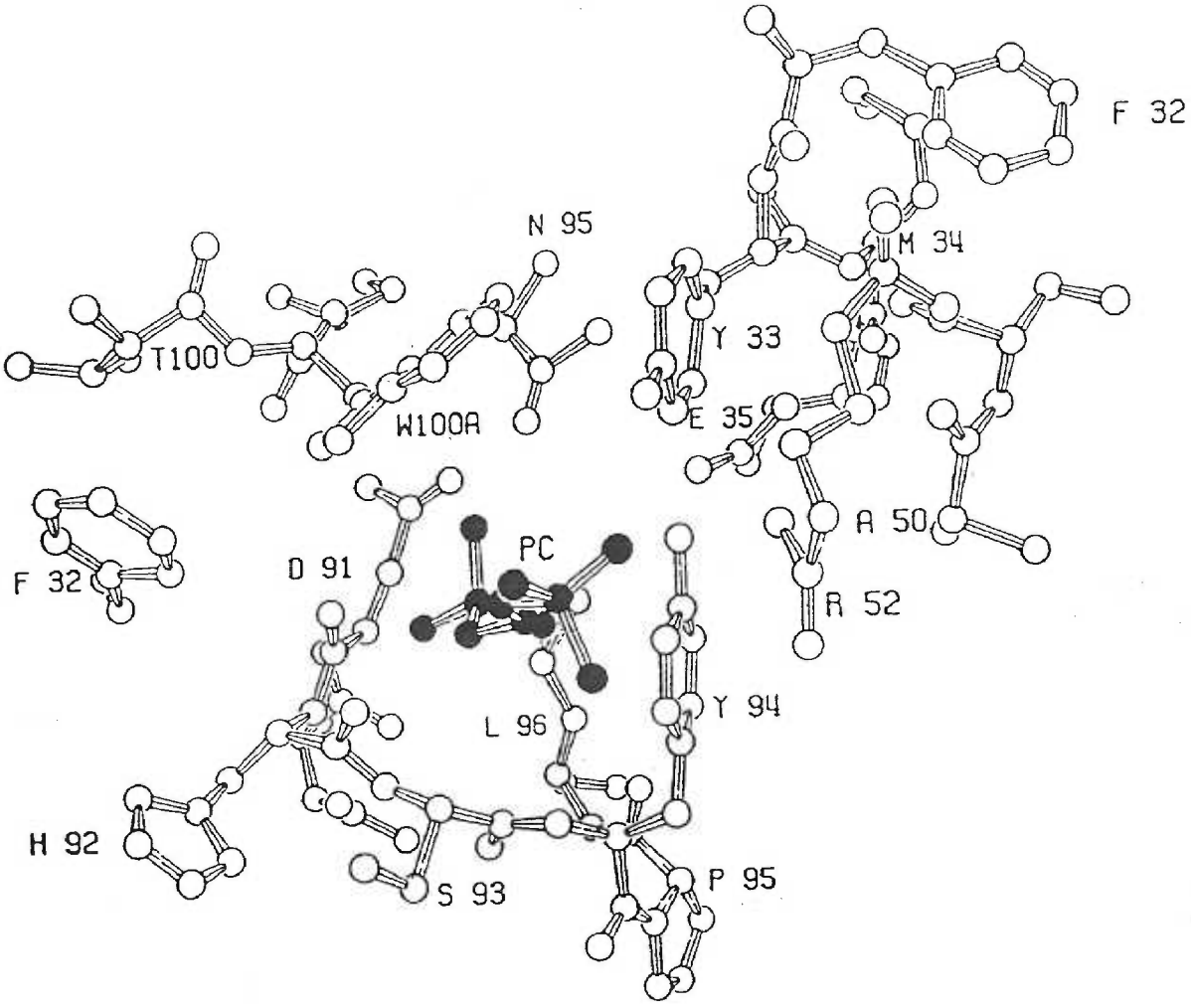


FIGURE 3. McPC603 binding site. Heavy chain complementarity-determining regions are located in the upper portion. Light chain contributions are mainly from CDR3-L (lower portion). PC hapten is indicated by solid circles. (Taken from Rudikoff 1983).


F 32
N 95
M 34
T100
Y 33
W100A
E 35
A 50
F 32
D 91
PC
R 52
Y 94
L 96
H 92
S 93
P 95

References

Bernard, O., and Gough, N. M. (1980) Nucleotide sequence of immunoglobulin heavy chain joining segments between translocated VH and mu constant regions genes. Proc. Natl. Acad. Sci. USA 77(6):3630-4.

Brack, C., Hirama, M., Lenhard-Schuller R., and Tonegawa, S. (1978) A complete immunoglobulin gene is created by somatic recombination. Cell 15(1):1-14.

Briles, D. E., Nahm, M., Schroer, K., Davies, J., Baker, P., Kearney, J., and Barletta, R. (1981) Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. J. Exp. Med. 153(3):694-705.

Chang, S. P., Brown, M., and Rittenberg, M. B. (1982) Immunologic memory to phosphorylcholine. II. PC-KLH Induces two antibody populations that dominate different isotypes. J. Immunol. 128(2):702-706.

Chang, S. P., Perlmutter, R. M., Brown, M., Heusser, C. H., Hood, L., and Rittenberg, M. B. (1984) Immunologic memory to phosphocholine. IV. Hybridomas representative of group I (T15-like) and group II (non-T15-like) antibodies utilize distinct VH genes. J. Immunol. 132(3):1550-1555.

Chen, C., Roberts, V. A., and Rittenberg, M. B. (1992) Generation and analysis of random point mutations in an antibody CDR2 sequence: many mutated antibodies lose their ability to bind antigen. J. Exp. Med. 176:855-866.

Claflin, J. L. (1976) Uniformity in the clonal repertoire for the immune response to phosphorylcholine in mice. Eur. J. Immunol. 6(10):669-74.

Clarke, S. H., Claflin, J. L., Potter, M., and Rudikoff, S. (1983) Polymorphysms in anti-phosphocholine antibodies reflecting evolution of immunoglobulin families. J. Exp. Med. 157(1):98-113.

Crews, S., Griffin, J., Huang, H., Calame, K., and Hood, L. (1981) A single VH gene segment encodes the immune response to phosphocholine: somatic mutation is correlated with the class of the antibody. Cell 25(1):59-66.

Davies, D. R., Padlan, E. A., and Segal, D. M. (1975) In: *Contemporary Topics in Molecular Immunology*, Volume 4 (Inman, F. P., and Mandy, W. J., eds.), pp. 127-155, Plenum Press, New York.

Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980) An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. Cell 19(4):981-92.

Golub, E. S., and Green, D. R. (1991) *Immunology a Synthesis*. (2nd edition) Sinauer Associates, Inc. Massachusetts.

Honjo, T., Kataoka, T. (1978) Organization of immunoglobulin heavy chain genes and allelic deletion model. Proc. Natl. Acad. Sci. USA 75(5):2140-4.

Kurosawa, Y., and Tonegawa, S. (1982) Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. J. Exp. Med. 155(1):201-18.

Lieberman, R., Potter, M., Mushinski, E. M., Humphrey, W., and Rudikoff, S. (1974) Genetics of a new IgVH (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. J. Exp. Med. 139(4):983-1001

Max, E. E., Seidman, J. G., and Leder, P. (1979) Sequences of five potential recombination sites encoded close to an immunoglobulin kappa constant region gene. Proc. Natl. Acad. Sci. USA 76(7):3450-4.

Padlan, E. (1994) Anatomy of the antibody molecule. Mol. Immunol. 31(3):169-217.

Padlan, E. A., Davies, D. R., Rudikoff, S., and Potter, M. (1976) Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. Immunochem. 13:945-949.

Padlan, E. A., Cohen, G. H., and Davies, D. R. (1985) On the specificity of antibody/antigen interactions: phosphochoine binding to McPC603 and the correlation of three-dimensional structure and sequence data. Ann. Inst. Pasteur. Immunol. 136C(2) 271-276.

Perlmutter, R. M., Crews, S. T., Douglas, R., Sorensen, G., Johnson, N., Nivera, N., Gearhart, P. J., and Hood, L. (1984) The generation of diversity in phosphorylcholine-binding antibodies. Adv. Immunol. 35:1-37.

Pèry, P., Petit, A., Poulian, J., and Luffan, G. (1974) Phosphorylcholine-bearing components in homogenates of nematodes. Eur. J. Immunol. 4(9):637-9.

Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizackerley, R. P., and Saul, F. (1973) Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2.8-Å resolution. Proc. Natn. Acad. Sci. USA 70:3305-10.

Potter, M. (1971) Antigen-binding myeloma proteins in mice. Ann. N. Y. Acad. Sci. 190:306-21

Rabbitts, T. H., Forster, A., Dunnick, W., and Bentley, D. L. (1980) The role of gene deletion in the immunoglobulin heavy chain switch. Nature 283(5745):351-6.

Rao, D. N., Rudikoff, S., Krutzsch, H. and Potter, M. (1979) Structural evidence for independent joining region gene in immunoglobulin heavy chains from anti-galactan myeloma proteins and its potential role in generating diversity in complementarity-determining regions. Proc. Natl. Acad. Sci. USA 76(6):2890-4.

Rudikoff, S. (1983) Immunoglobulin structure-function correlates: antigen binding and idiotypes. Contemp. Topics Mol. Immunol. 9:169-209.

Sakano, H., Kurosawa, Y., Weigert, M., and Tonegawa, S. (1981) Identification and nucleotide sequence of a diversity DNA segment (D) of immunoglobulin heavy-chain genes. Nature 290(5807):562-5.

Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980) Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. Nature 286(5774):676-83.

Sakano, H., Huppi, K., Heinrich, G., and Tonegawa, S. (1979) Sequences at the somatic recombination sites of immunoglobulin light-chain genes. Nature 280(5720):288-94.

Schilling, J., Clevinger, B., Davie, J. M., and Hood, L. (1980) Amino acid sequence of homogeneous antibodies to dextran and DNA rearrangements in heavy chain V-region gene segments. Nature 283(5742):35-40.

Sher, A., and Cohn, M. (1972) Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. Eur. J. Immunol. 2(4):319-26.

Shimizu, A., Takahashi, N., Yamawaki-Kataoka, Y., Nishida, Y., Kataoka, T., and Honjo, T. (1981) Ordering of mouse immunoglobulin heavy chain genes by molecular cloning. Nature 289(5794):149-53.

Stenzel-Poore, M. P., Bruderer, U., and Rittenberg, M. B. (1988) The adaptive potential of the memory response: clonal recruitment and epitope recognition. Immunol. Rev. 105:113-136.

Stenzel-Poore, M. P., and Rittenberg, M. B. (1989) Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. J. Immunol. 143(12):4123-4133.

Stenzel-Poore, M. P., and Rittenberg, M. B. (1987) V gene diversity in the memory immune response to PC-KLH. In *H-2 Antigens*, (Edited by Chells, S. D.), pp. 827-835. Plenum Publishing Corporation. New York.

Tomasz, A. (1967) Choline in the cell wall of a bacterium: novel type of polymer-linked choline in *Pneumococcus*. Science 157(789):694-7.

Weigert, M., Gatmaitan, L., Loh, E., Schilling, J., and Hood, L. (1978) Rearrangement of genetic information may produce immunoglobulin diversity. Nature 276(5690):785-90.

Wright, A., Seung-Uon, S., and Morrison, S. L. (1992) Genetically engineered antibodies: progress and prospects. Crit. Rev. Immunol. 12(3,4):125-168.

Wu, T. T., and Kabat, E. A. (1970) An analysis of the sequences of the variable regions of Bence-Jones proteins and myeloma light chains and their implication for antibody complementarity. J. Exp Med. 132:211-49.

Yaoita, Y., and Honjo, T. (1980) Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome. Nature 286(5776):850-3.

TITLE :

Site-directed Mutagenesis of L96 of Primary and Secondary Immune Response Antibodies Against PC-KLH: A Critical Residue in Primary Rather Than in Secondary Antibody.

Arun Bhat†, Tammy M. Martin†, and Marvin B. Rittenberg†

†Oregon Health Sciences University

Department of Molecular Microbiology and Immunology

Mail Code L220

3181 S. W. Sam Jackson Park Road

Portland, OR 97201

INTRODUCTION

X-ray crystallography has been a powerful tool for identifying functionally important features of the antibody combining site (reviewed by Padlan 1994). Many of the residues in the complementarity determining regions (CDRs) that make contact with antigen have been distinguished from other residues important in maintaining the conformational integrity of the binding site. In the absence of crystallographic data for a defined antigen-antibody model system, potential contact and canonical residues have been inferred by examining V gene sequences from a number of unrelated antibodies directed against the antigen under study. Residues occurring more frequently at a specific position in the V sequence suggested these residues as being important in antigen binding and/or integrity of the binding site (Kabat et al., 1991).

The immune response to phosphocholine (PC)-protein is characterized by two distinct antibody populations (Chang et al., 1982; Wicker et al., 1982; and Ettinger et al., 1982). In the primary response, referred to as Group I, anti-PC antibodies which are T15 idiotype (id) positive, represent > 90% of the antibody (Claflin and Rudikoff 1977; Cosenza and Kohler 1972; and Quintans and Cosenza 1976). Upon secondary immunization, the memory population, referred to as Group II, which is T15 id negative, recognizes a distinct epitope (phenyl-PC) that includes the phenyl linkage to the carrier protein (Bruderer et al., 1989; Chang et al., 1982, 1984; Stenzel-Poore et al., 1987, 1988, 1989, 1991; Wicker et al., 1982).

The understanding of how PC fits into the binding site came from the analysis of the crystal structure of McPC603, a myeloma protein whose crystal structure was determined well before sequence data was available for many antibody V regions (Padlan et al., 1976, 1985, 1994; Satow et al., 1986; Segal et al., 1974). Upon

availability of these data it was found that Group I antibodies predominantly used the S107, VH1 gene together with 3 VLs, κ8, κ22 and κ24; κ22 being the light chain of T15 antibodies and therefore used in more than 90% of cases during the primary response (Claflin and Rudikoff 1977; Perlmutter 1984). It is generally accepted that V genes code for residues 1-95 and the J genes code for residues 96-108 in most κ L chains in the absence of any insertions or deletions introduced during gene rearrangement (Kabat et al., 1991). Analysis of the VL sequences from Group I antibodies showed that leucine was present at residue 96 in all cases (Kabat et at., 1991; Rudikoff et al., 1981). Jκ5 was the predominant J gene used in these antibodies ; Jκ5 codes for a leucine at position 96. Jκ5 is the only germline J gene encoding a non-aromatic amino acid at this position (Kabat et al., 1991). In cases where Group I light chain was found to rearrange to a different J gene, which would normally encode an aromatic amino acid at position 96, this residue was often mutated to a leucine (Kabat et al., 1991; Lotscher et al., 1990). From the sequence data of Group I antibodies the occurrence of a leucine at position 96 in the light chain suggested this residue may be involved either in binding to phosphocholine or maintaining the integrity of the binding site in Group I antibodies. Padlan et al., (1976) suggested from the crystal structure that the binding site of McPC603 was a deep cavity. PC was found to bind in this cavity with the choline group buried deep in the interior and the phosphate moiety more towards the exterior of the cavity. The positively-charged trimethylammonium moiety of the hapten interacted with the acidic side chains of Glu H35 and Glu H59, whereas the negatively charged phosphate group interacted with the positively charged guanidium group of Arg H52 and Lys H54. Extensive van der Waals contacts existed between the hapten and the side chain of Tyr H33, Trp H104a and Leu L96. From this three dimensional analysis L96 was modeled to be at the bottom of the phosphocholine binding cavity. Considering the model it was speculated that if residue 96 were mutated to an amino acid with a bulky side chain like an aromatic

amino acid, the binding of the hapten would be decreased or abolished due to steric effects (Rudikoff et al., 1981). This hypothesis was tested by Glockshuber, et al., (1991) who mutated residue 96 from a leucine to either a Phe or Tyr in the McPC603 F_V fragment. Mutating this residue to an aromatic amino acid, as predicted, abolished binding of the F_V fragment to PC-sepharose columns due probably to steric hindrance. In this paper we have extended the work of Glockshuber et al., (1991) to the T15 molecule which differs from McPC603 at four VH residues and uses a different VL but retains Leu at position L96. In addition we have addressed whether VL residue 96 may be important in distinguishing the Group II binding phenotype characteristic of the Group II anti-phenyl PC antibodies characteristically found in the memory response to PC-protein.

In the Group II memory response antibodies against PC-KLH generated during secondary immunization, there is diverse usage of VH and VL genes and a higher level of somatic mutations found in the V region compared to T15 (Bruderer et al., 1989; Chang et al., 1982, 1984; Stenzel-Poore et al., 1987, 1988, 1989, 1991). It has been speculated from previous investigations that the affinity maturation of Group II antibodies is directed much more towards the carrier than the PC moiety (Stenzel-Poore and Rittenberg, 1991). Upon examining the V region sequences from a number of different Group II antibodies using both lambda and kappa light chains, we found that residue 96, unlike Group I antibodies, was predominantly an aromatic amino acid; probably due to their use of Jκ and Jλ elements encoding an aromatic amino acid at this position (Stenzel-Poore and Rittenberg, 1991). In cases where the rearrangement had occurred to Jκ5 (encoding a leucine), this residue usually was mutated to an aromatic amino acid (Stenzel-Poore and Rittenberg, 1989) or a Val in one case (Lotscher et al., 1993). The frequent occurrence of an aromatic amino acid instead of a leucine at this position suggested that this residue may be important in creating the specificity seen in

these antibodies for the phenyl-PC moiety, specifically the phenyl linkage to the carrier protein. Secondly as this residue lies at the center of the binding site in Group I antibodies we wondered whether it was critical in distinguishing the Group I antigen-binding fine specificity from Group II, that is, would mutating this residue to a leucine confer the Group I binding character on a Group II antibody. And lastly would mutating position 96 from a leucine to an aromatic amino acid in the prototypical Group I antibody, T15, using the predominant κ22 light chain abolish binding as it does in McPC603 which uses κ8 (Glockshuber, et al., 1991).

Using site-directed mutagenesis, we found that changing L96 in T15 (κ22) to an aromatic amino acid abolished its binding to PC whereas in a prototypic Group II antibody M3C.65 (λ-1) changing the aromatic amino acid at position 96 to a leucine did not appreciably effect the Group II fine specificity character but did affect the affinity of the antibody.

MATERIAL AND METHODS

Plasmids and vectors. pTZ18U phagemid containing the F1 ori that allows production of single stranded DNA needed for site-directed mutagenesis was obtained from Bio-Rad Laboratories (Richmond, CA). For the T15 antibody the genomic clone of S107 VH1 gene and pSV2*neo*Vκ22Cκ constructs were kindly provided by Dr. Sherie L. Morrison (University of California, Los Angeles, CA) (Morrison et al., 1984). pSV2γ2b was a kind gift from Dr. J. Sharon (Boston University School of Medicine) (Sharon et al., 1989). The pSV2*gpt*S107γ2b vector and pSV2*gpt*M3C.65γ2b containing the VH from M3C.65 and the VL placed in pTZ18 were constructed in our lab as described previously (Brown et al., 1992). For production of single stranded DNA and subsequent site-directed mutagenesis Vκ22Cκ (genomic) was cloned into pTZ18U. pSV2*neo*Vκ22Cκ was restriction digested with BamH1 releasing the 7 kb BamH1 Vκ22Cκ fragment which was subsequently cloned into BamH1 digested and phosphatase (CIP) treated pTZ18U vector giving pTZ18UVκ22Cκ.

Antibody site-directed mutagenesis. Mutagenesis was performed in the pTZ18U phagemid using the Muta-Gene phagemid mutagenesis kit (Bio-Rad, Richmond, CA). Oligonucleotides containing the desired substitutions were synthesized by Research Genetics (Huntsville, AL) or in the laboratory of J.P. Adelman (Vollum Institute, Portland, OR). The oligonucleotides used to mutate residue 96 in the T15 light chain from a leucine to an aromatic amino acid were: (L96F) 5' AGC TAT CCT *TTC* ACG TTC G 3', (L96Y) 5' TAC AGC TAT CCT *TAC* ACG TTC GGT GCT 3' and (L96W) 5' TAC AGC TAT CCT *TGG* ACG TTC GGT GCT 3'. For M3C.65 the oligonucleotide for mutating residue 96 from Trp to Leu was (W96L) 5' TAC AGC AAC CAT *TTG* GTG TTC GGT GGA 3'. Codons for position 96 are in italics and nucleotide changes are underlined. The mutations were verified by dideoxynucleotide

chain termination sequencing on dsDNA purified from phagemid grown in *Escherichia coli* XL1-Blue using the Sequenase kit (USB, Cleveland, OH).

Transfectoma production. The H chain constructs were in the pSV2*gpt* vector, and the L chain constructs used for cotransfection were in pTZ18U (in which case selection for the *gpt* marker was based on expression of Gpt by the H chain construct). T15 uses the germline VH1 S107. Whereas MC3.65 uses VH M141 which was cloned in our laboratory and is derived from the germline gene, pj14 a member of the Q52 VH family (Stenzel-Poore et al., 1987). MC3.65 has the following somatically mutated residues in the heavy chain: Ala H68, Gly H73, Gly H81, Tyr H82a, and Tyr H93; the light chain mutated residues are: Lys L52, His L53, and Tyr L55. The protocol for transfection of SP2/0 monolayer cultures using Lipofectin Reagent (BRL, Gaithersburg, MD) was followed. After transfection the cells were selected in Iscove's modified MEM with 20% FCS and HMX (15μg/ml hypoxanthine, 6μg/ml mycophenolic acid, 250μg/ml xanthine) for 6-14 days, screened and subsequently transferred into medium containing no selection drugs.

Antibody purification. Antibody secreted by transfectomas was purified from tissue culture supernatants via affinity chromatography on PC-sepharose 4B as described previously (Brown et al., 1992; Chang et al., 1984), except that antibodies were eluted from the column with 0.1 M NPPC. For T15 L96 aromatic amino acid mutants, Protein A affinity purification procedure was followed as described (Brown et al., 1992; Chang et al., 1984).

ELISA. Direct binding and solid phase inhibition ELISA were used as described previously (Brown et al., 1992; and Chang et al., 1984). For screening transfectomas for secretion of antibody, 10-15 days after transfection supernatants from 96 well plates

were screened with goat anti-mouse IgG, goat anti-mouse κ and PC-histone binding. Cells secreting γ and κ chains were expanded and saved. Expression of the T15 idiotype was tested with three different mabs and anti-T15 antiserum. A goat anti-T15 antiserum was kindly provided by Dr. J. Kenny (NCI). This antiserum is specific for the T15 VH-VL conformation. The three mabs were AB1-2, B36-82 and TC54. Mab B36-82 (γ1, κ) (Cerny, et al., 1982; and Strickland, et al., 1987), was a kind gift from Dr. J. Cerny (University of Maryland, Baltimore, MD) and reacts with T15 VH-VL. The recognition of T15 by B36-82 is not inhibited by free PC and is only marginally inhibited by PC-BSA. TC-54 detects residues on VH S107 only. Residues important for binding to this antibody are in the CDR2 (Chen et al., paper submitted). For all anti-id testing, purified anti-T15 antibody was coated on the ELISA plates and then overlaid with purified mutant T15 antibodies at 100 ng/ml. Binding was detected by further incubation with rabbit anti-mouse γ2b antibody coupled to alkaline phosphatase and developed with the substrate, nitrophenyl phosphate. Wells receiving buffer instead of T15 antibody were used as blanks. The T15 L96 aromatic amino acid mutants were also screened for binding to various PC-unrelated antigens, including BSA, histone, KLH, albumin, ubiquitin, actin, FITC-BSA, DNP-BSA, oxazolone-BSA, soman-BSA, and ssDNA as described previously. The I_{50} values for inhibitors PC, p-nitrophenyl PC (NPPC), L-α-glycero-PC (GPC), PC-BSA were determined as described previously (Chang 1982, 1984).

RESULTS

On examining a number of Group I and Group II light chains (Table I) we found that residue 96 in either λ or κ Group I L chains was always a leucine, whereas in most Group II antibodies this residue was an aromatic amino acid. We previously found two Group II clones G1-14 and 52-1 (Stenzel-Poore and Rittenberg, 1989) that utilized Jκ5 normally coding for a leucine at position 96, however in the case of these two antibodies the first codon of the J gene was mutated from the Leu to a Phe in the case of G1-14 and either a Phe or Val in 52-1. Lotscher et al., (1990) also found residue 96 in Group II antibodies to be predominantly an aromatic amino acid though there was one Group II antibody, aPC-111-6 that utilized a Val at this position. The predominance of an aromatic amino acid at this position suggested that Group II antibodies may have a requirement for an aromatic amino acid similar to the requirement for a non-aromatic amino acid in Group I antibodies and that this residue may be important in conferring the fine specificity differences between Group I and Group II antibodies. This hypothesis was also based on the view that light chains are important in the formation of antibody combining sites that bind aromatic ring structures (Buenafe et al., 1989) and that in Group II antibodies, λ light chains play a much more significant role than H chain in binding to PC-phenyl-haptens (Brown et al., 1992; Stenzel-Poore et al., 1988, 1989).

Site-directed mutagenesis of light chains from T15 and M3C.65

T15 and M3C.65 were chosen as prototypical Group I and Group II antibodies. Both antibodies show high relative affinities for NPPC as determined by I_{50} measurements: T15 (0.036mM) (Chang et al., 1984) and M3C65 (0.19 mM) (Brown et al., 1992). Mutagenesis of position L96 was carried out according to the manufacturers protocol (Bio-Rad Laboratories). Fig.1a shows the mutated residue in T15 Vκ22. This residue

was mutated to an aromatic amino acid, Phe (Jκ4), or Tyr (Jκ2) or Trp (Jκ1), whereas Fig.1b shows M3C.65 VL residue 96 mutated from a Trp (Jκ1) to Leu (Jκ5), characteristic of group I antibodies; other mutations in M3C.65 CDR2 at residues 52, 53 and 55 had previously been shown to be critical for improved binding to NPPC (Brown et al., 1992).

Fine specificites of T15

After cotransfection of VH and VL into SP2/O non-secreting myeloma cells, transfectomas were selected using HMX as the selection was on H chain; cells receiving H chain DNA generated drug resistant colonies. 10-15 days later these colonies were screened for secretion of assembled antibody by a sandwich ELISA. Amongst the total number of HMX resistant clones some Group I mutants were found to secrete assembled antibody but this antibody did not bind PC-histone in an ELISA. Three clones from each mutant were expanded and saved. The supernatants from these clones were affinity purified using Protein A and the protein eluted and quantitated for further binding studies. Purified antibody from the T15 mutants did not bind PC-histone in an ELISA when examined at up to 2μg/ml (data not shown). The mutant proteins at this concentration did not bind to other PC-conjugates either, i.e., PC-BSA, PC-KLH, and PC-carbohydrate, (R36a bacterial cell walls in which PC is attached to the carbohydrate via a teichoic acid linkage) (data not shown). The lack of recognition of the PC-moieties is consistent with previous results with McPC603 supporting the role of L96 as a key feature of the T15 combining site and presumably of all Group I antibodies.

The lack of binding could have resulted from localized damage within the binding site or from a more global conformational disturbance. To test this latter possibility we used three anti-id mabs (AB1-2, B36-82 or TC54) and an anti-T15 polyclonal

antiserum. All of these anti-ids except TC54 recognize an epitope made up of the combination of H and L chains together. TC54 is VH1 specific and we have recently found that VH1 CDR2 residues Lys H52b, Asp H54, Tyr H55, Thr H57, Tyr H59 and Ile H68, provide a solvent exposed surface that is likely to encode the TC54 epitope (Chen et al., paper submitted). We found reactivity of all three anti-id mabs (AB1-2, B36-82 or TC54) and the anti-T15 antiserum, towards the mutants and the wild type T15 antibody to be indistinguishable, at all concentrations tested (data not shown). These data suggest the global conformation of the mutant antibodies was likely to be intact indicating the loss of binding in the mutants most likely resulted from a more local perturbation in the structure of the combining site.

Giusti et al., (1987) showed substitution of Ala H35 for Glu in the H chain V region of T15 resulted in the antibody acquiring new specificity, namely, anti-DNA. We asked whether altering residue 96 of the light chain, might have a similar effect and result in binding to a different antigen unrelated to PC. We therefore examined the binding of all three T15 L96 mutants (Leu96Trp, Leu96Tyr, and Leu96Phe) against a panel of antigens by ELISA. The following antigens were tested: BSA, histone, KLH, albumin, ubiquitin, actin, FITC-BSA, DNP-BSA, oxazolone-BSA, soman-BSA, and ssDNA. None of the mutant antibodies bound any of the above antigens (data not shown). Thus there was no evidence that introducing an aromatic amino acid at L96 would be capable of altering specificity.

Fine specificity of M3C.65 mutated at L96

Co-transfection of H and L chain of M3C.65 wt and mutants resulted in transfectomas secreting antibody that bound PC-histone on the primary screen. These clones were expanded, saved and the antibody purified. As the mutants bound PC, we wanted to determine whether there was a substantial change in affinity of the antibody compared

to wild type. Relative affinities (I_{50}) were determined using inhibition ELISA of the wt and the mutants. Two clones were picked for further analysis (M3C65W96L.1 and M3C65W96L.9). Table 2 shows the I_{50} values for 4 different PC analogs. We found that M3C.65 wt or mutants did not bind to PC or GPC consistent with their Group II character, i.e., not being inhibited by these analogs as these haptens do not possess the phenyl phosphate linkage essential for Group II binding (Chang et al., 1982). There was a 7 fold affinity increase for NPPC between the mutants and wt and 3.6 fold increase for NPDBP, whereas for PC-BSA there was a 20 fold decrease in affinity. These results show that Group II binding in these mutants does not shift to Group I when residue 96 is changed from Trp to Leu and that the change in this single amino acid results in a modest increase in affinity for small PC analogs as compared to an overall decrease in affinity for PC-protein.

DISCUSSION

In this paper we examined the importance of position 96 of the L chain in Group I and Group II anti-PC-protein antibodies. All Group I antibodies contain leucine at L96 while Group II antibodies predominantly possess an aromatic amino acid at this position. Examination of this residue in the crystal structure of McPC603 (Padlan et al., 1976; Rudikoff et al., 1981) and a model of the T15 molecule (Chien et al., 1989) revealed its position is at the center of the binding site. We inferred that L96 may be responsible for conferring the Group I and Group II binding phenotypes to these antibodies. This view is consistent with previous work showing that mutating L96 in McPC603 from Leu to either a Trp or Tyr, abolished binding of the antibody to PC (Glockshuber et al., 1991). We have extended that work to another Group I antibody, T15, which uses a different L chain than McPC603 and differs at four residues in the VH1 sequence. We report here that mutating L96 in T15 destroyed its binding to PC in keeping with Glockshuber's work. In contrast when L96 in M3C.65, a Group II antibody, was mutated from Trp to Leu, the Group II binding phenotype was unchanged, however there was a significant change in affinity for PC-protein. Thus it appears that this residue is a critical feature in antigen binding of group I antibodies although it does not represent a residue that solely confers the group I or group II phenotype as determined by fine specificity binding studies.

Residue 96 of the light chain is at the junction of VJ joining that occurs during gene recombination in the developing B cell. Although germline J genes code for either an aromatic amino acid or a leucine, one sees more variability at this position than can be accounted for by the germline genes. This variability arises from imperfect VJ joining (junctional diversity) and addition or deletion of nucleotides due to somatic mutation (Manser et al., 1987; Jeske et al., 1984; Pawlita et al., 1982). Pawlita et al., (1982)

have even suggested the use of intronic sequences coding for residue L96 in the anti-galactan antibody response; the residue at this position often being an Ile. On examination of other defined antigen systems VL 96 has often been shown to be critical for binding. In the anti-oxazolone response as in the Group I response to PC, there is pressure to utilize Jκ5 which provides the Leu at position 96 (Berek and Milstein, 1987). Similarly in the anti-azophenylarsonate response position 96 seems usually to be an Arg arising from the imprecise joining of V and J genes (Jeske et al., 1984; and Manser et al., 1987). Not only has L96 been shown to be critical for binding to antigen, it has also been shown to play a critical role in catalysis; in the antibody NPN43C9 which accelerates amide hydrolysis by a factor of 10^6, Arg L96 appears to function both in binding and catalysis (Stewart et al., 1994; and Roberts et al., 1994). This residue in the light chain is also important in the binding to a sweetener (NC174); in this case a Tyr at L96 is essential for immobilizing the antigen in the binding site, prior to induction of a conformational change necessary for optimal binding of the sweetner (Guddat et al., 1994). Similarly the presence of an aromatic amino acid at L96 was found to be important for affininty for another aromatic hapten, fluorescein; when L96 was a Trp the antibody had an intermediate affinity approx. 10^7 M^{-1} whereas if this was substituted by a Leu, the affinity dropped drastically by about 1000 fold (Bedzyk and Voss, 1991). The crystal structure of antifluorescein Fab 4-4-20 showed that the L96 Trp made contact with the xanthenone moiety of fluorescein (Herron et al., 1989). In digioxinin binding, mutating L96 from Pro to Leu in the context of the H chain caused a total loss of binding to the hapten (Hudson et al., 1990). The pressure to maintain a certain residue at L96 may arise through a direct, important contribution to antigen binding or to the spatial relationships within the site.

McPC603 is a well characterized antibody that has been crystallized with (Segal et al., 1974) and without (Satow et al., 1986) bound PC and provides an excellent model to

examine Group I antibody binding. From this model it has been shown that charged residues in the binding cavity of the antibody are essential for binding to hapten. The orientation of PC in the active site is determined by binding of the phosphate and trimethylammonium groups. From the T15 model (based on the McPC603 crystal structure) (Chien et al., 1989) the positively charged quaternary ammonium group of PC is bound by electrostatic and van der Waals forces within a pocket at the bottom of the binding site formed by the CDR3 of the H and L chain. Residues Phe L91, Tyr L94 and Leu L96 and Asp H95 and Trp H100B provide the residues that make this pocket containing the choline. Although there are no direct salt-bridges or H-bonding with the positively charged trimethylammonium of choline, the positive charge on the N is neutralized by a de-localized negative charge provided by the side groups of residues lining the pocket. The phosphate moiety is oriented more towards the lip of the binding site and is partially exposed to solvent. This negatively charged group makes an essential salt-bridge contact with Arg H52. To maintain the binding site however, there is an extensive hydrogen bonding network surrounding the choline which involves Glu H35 H-bonding to Tyr L94 and Asp H95 (Chien et al., 1989; and Chen et al., 1992).

The T15 model has previously been used to examine the mechanism by which point mutations in the H chain can lead to a non-binding antibody. Binding to PC was abolished when residues at two H chain positions were spontaneously mutated in S107 hybridomas which produce T15 antibody (Kobrin et al., 1991; and Chien et al., 1989). The anti-phosphocholine cell line PC700 (IgM) which utilizes germline S107 VH and κ22 (T15 id$^+$) contains a mutation of Asp H95 to Asn and no longer binds PC. Similarly another hybridoma, U10, lost binding after a single amino acid substitution of Asp H101 with Ala (Kobrin et al., 1991; and Chien et al., 1989). Thus residues 95 and 101 play crucial role in the heavy chain's contribution to binding PC. Neither of these two mutations directly interacted with the hapten as shown by modeling. Both

were found to be essential in maintaining the binding site of the antibody, the mutation at position 101 in fact was 9Å away from the hapten (Chien et al., 1989). On probing the binding site with anti-id mabs they found the changes to be local, rather than global in nature. Thus in accord with our results certain residues are heavily constrained in the T15 binding site and any mutation at these sites may result in a non-binding phenotype; thus this loss of binding results not from drastic global alteration of the binding site but more likely from subtle local changes.

The prototypical antibody, T15, with its specificity for the phosphate-containing PC can be converted to bind another phosphate containing polymer, DNA, via a single amino acid change in the VH (Giusti et al., 1987). In this case the mutation of Glu H35 to Ala converted the specificity for PC to DNA. Glu35 does not make contact with PC directly but is essential in making a H-bond with Tyr 94 of the VL which helps stabilize the conformation of the combining site (Giusti et al., 1987; and Padlan 1976, 1985). Similarly we questioned whether mutating L96 would also alter the specificity of T15. When we tested the binding of the mutated T15 against a panel of 14 non-PC related antigens we did not detect any new specificities. This is consistent with the proposal of Malipiero et al., (1987) that the physiologic function of somatic mutation is to increase the affinity of antibodies rather than to extend the basic repertoire and that altered specificity is likely to be a rare event.

The inability of T15 to tolerate an aromatic residue at L96 further supports the view that T15 is evolutionarily well suited for PC binding and poorly suited for further diversification.

On the other hand in M3C.65 a prototypical Group II antibody that uses a λ1 L chain, the binding site contains some important differences from the residues found to be

crucial in the T15 model. Residues that were important in forming the binding pocket for choline in T15, are different in M3C.65. L91 is a Phe in T15 but a Trp in M3C.65; L94 is a Tyr in T15 but an Asp in M3C.65; and L96 is a Leu in T15 but Trp in M3C.65. Similarly there are differences in the VH CDR3 : H95 is an Asp in T15 but a Gly in M3C.65; and H98 is a Gly in T15 but a Pro in M3C.65 (Brown et al., 1992; Clarke et al., 1983). This suggests that PC bound much differently in M3C.65 as compared to T15. We have recently modeled another Group II antibody D16 which uses the same VH as T15 but a different VL (κ1-C) coding for a Trp at L96. This binding pocket is quite different from the T15 binding pocket due both to electrostatic and shape differences caused by different VL and VH CDR3 residues (Chen et al., paper submitted). In the D16 model the phosphate of PC binds differently than T15; the phosphate is deeper in the pocket and the choline is further removed from the L96. Due to the deeper binding of phosphate in D16, the possibility of contacts occurring with the carrier is suggested. This may explain why Group II antibodies are not inhibitable by PC, and have a higher affinity for PC analogs carrying a phenyl ring, e.g., NPPC, which resembles the immunizing form of the antigen (Chen et al., paper submitted).

From our inhibition data we found that in the M3C.65 mutants there was an increase in affinity for two haptens NPPC and NPDBP whereas the affinity for PC-BSA decreased considerably. The absence of binding to PC and GPC which do not possess the phenyl linkages important for Group II binding points to the mutants still retaining the Group II character. Both the wild type M3C.65 and the mutants showed binding to NPDBP which classified them as Group IIb antibodies (Bruderer et al., 1988). These antibodies unlike Group IIa antibodies which have a requirement for positively charged trimethylammonium in the choline moiety, can bind to NPDBP which has the N replaced with a C and thus the positive charge is lacking (Bruderer et al., 1988). The

binding for NPDBP was increased nearly 4 fold in the mutants suggesting that certain residues in the binding site of the mutants are positioned such that they can better accommodate choline as compared to wild type antibody. Leucine being a smaller amino acid with a volume of 166.7 $Å^3$ and an accessible surface area of 170 $Å^2$ as compared to Trp with volume 227.8 $Å^3$ and an accessible area of 255 $Å^2$ (Chothia 1976; and Zamyatnin 1972) may increase the overall volume of the binding cavity thus accommodating smaller PC related haptens. As the increase is not substantial, it suggests that the orientation of the hapten as it sits in the binding site in the mutants may not have changed substantially. The increase in affinity for NPPC being greater than for NPDBP is consistent with previous data which has shown that Group IIb antibodies which bind NPDBP, bind NPPC with increased affinity suggesting the charge on the N although not required for binding, nevertheless aids in stronger non-covalent interactions.

The observation that even though most Group II antibodies have an aromatic amino acid at position 96 perhaps due to the Jκ or Jλ gene used or due to somatic mutation, this residue is not critical for binding and may need to be maintained as an aromatic amino acid for other selective pressures, for example id, anti-id interactions. L96 in either Group I or Group II antibodies was also found not to be critical in conferring the fine binding specificities. This residue plays a much more critical role in maintaining a functional binding cavity in T15 antibodies as compared to a prototypical Group II antibody M3C.65.

Table I. Representative amino acids at position 96 of light chain in GroupI[a] and Group II[b] antibodies

	Number (total)[c]	Aromatics at L96[d]	Non-aromatics at L96[e]
Group I			
κ	20	0	20
λ	0	0	0
Group II			
κ	16	14	2
λ	22	22	0

[a]collated from the following references: Perlmutter et al., 1984; Padlan et al., 1985; Clarke et al., 1983; Lotscher et al., 1993; Kabat et al., 1991.

[b]collated from the following references: Lotscher et al., 1993; Brown et al., 1992; Stenzel-Poore and Rittenberg, 1989, 1991.

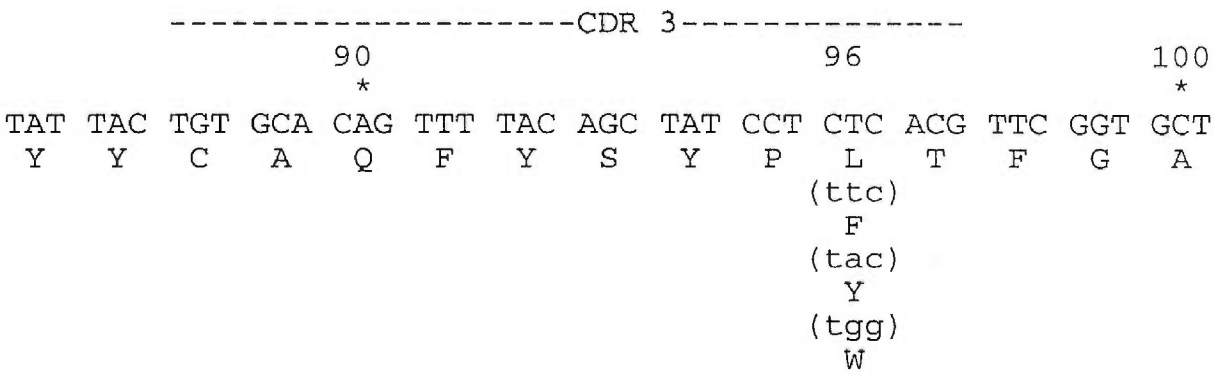
[c]number refers to clonally unrelated L chain sequences from different hybridomas

[d]aromatics refer to either a F, W, or Y

[e]non aromatics refers to any other residue than F, W, Y.

FIGURE 1. Nucleotide and amino acid sequence of (a) T15 light chain (κ22) and (b) MC3.65 light chain (λ1). Amino acid sequences were translated from nucleotide sequence and are given as single letter code. Amino acid residue numbering and CDRs are defined as per Kabat (1991). The codon change are shown in parenthesis and below these are the resulting replacement amino acids. Site-directed mutagenesis was performed on residue 96 in both (a) and (b). For (a) L96 was mutated to an aromatic (F, Y, or W). For (b) it was mutated to Leu only.

a)


CDR 3
90
96
100
TAT TAC TGT GCA CAG TTT TAC AGC TAT CCT CTC ACG TTC GGT GCT
Y Y C A Q F Y S Y P L T F G A
(ttc)
F
(tac)
Y
(tgg)
W

b)

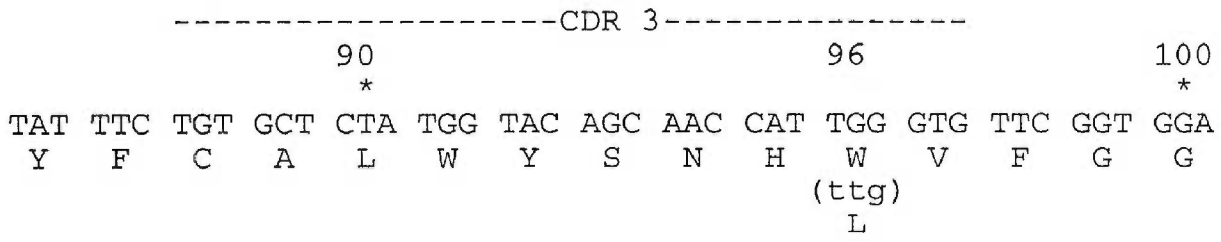

CDR 3
90
96
100
TAT TTC TGT GCT CTA TGG TAC AGC AAC CAT TGG GTG TTC GGT GGA
Y F C A L W Y S N H W V F G G
(ttg)
L

Table II. Binding properties of M3C.65 and mutant W96L clones.

Transfectoma	I_{50} Values (mM) PC	NPPC	GPC	NPDBP	PC-BSA ($X 10^{-7}$)
M3C.65	>10	0.218 ± 0.072	>10	1.280 ± 0.600	4.5 ± 1.4
M3C.65W96L.1	>10	0.032 ± 0.009	>10	0.340 ± 0.014	85 ± 4.2
M3C.65W96L.9	>10	0.029 ± 0.006	>10	0.375 ± 0.035	96 ± 6.0

M3C.65 mutants M3C.65W96L.1 and M3C.65W96L.9 result from separate transfection events.

I_{50} values are in mM of hapten required to achieve 50% inhibition of binding to PC-histone coated plates in an ELISA.

S.D. determined from an average of 2 to 5 experiments.

REFERENCES

Bedzyk, W. D., and Voss, E. W. Jr. (1991) Active site contributions to fluorescein binding determined by in vitro heavy and light chain reassociations. Mol. Immunol. 28(1-2):27-34.

Berek, C., and Milstein, C. (1987) Mutation drift and repertoire shift in the maturation of the immune response. 96:23-41.

Brown, M., Stenzel-Poore, M. P., Stevens, S., Kondoleon, S. K., Ng, J., Bachinger, H. P., and Rittenberg, M. B. (1992) Immunological memory to phosphocholine keyhole limpet hemocyanin, recurrent mutations in the λ1 light chain increase affinity for antigen. J. Immunol. 148(2):339-346.

Bruderer, U., Aebersold, R., Blaser, C., and Heusser, C. H. (1988) Characterization of the Group I and Group II antibody response against PC-KLH in normal and T15 idiotype-suppressed BALB/c mice. Immunol 64:385-90

Bruderer, U., Stenzel-Poore, M. P., Bachinger, H. P., Fellman, J. H., and Rittenberg, M. B. (1989) Antibody combining site heterogeneity within the response to phosphocholine-keyhole limpet hemocyanin. Mol. Immunol. 26(1):63-71.

Buenafe, A. C., Makowski, F. F., and Rittenberg, M. B. (1989) Molecular analysis and fine specificity of antibodies against an organophosphorus hapten. J. Immunol. 143(2):539-45.

Cerny, J., Wallach, R., and Hammerling, G. J. (1982) Analysis of T15 idiotopes by monoclonal antibodies: variability of idiotypic expression on phosphorylcholine-specific lymphocytes from individual inbred mice. J. Immunol. 128:1885.

Chang, S. P., Brown, M., and Rittenberg, M. B. (1982) Immunologic memory to phosphorylcholine. II. PC-KLH Induces two antibody populations that dominate different isotypes. J. Immunol. 128(2):702-706.

Chang, S. P., Perlmutter, R. M., Brown, M., Heusser, C. H., Hood, L., and Rittenberg, M. B. (1984) Immunologic memory to phosphocholine. IV. Hybridomas representative of group I (T15-like) and group II (non-T15-like) antibodies utilize distinct VH genes. J. Immunol. 132(3):1550-1555.

Chen, C., Roberts, V. A., and Rittenberg, M. B. (1992) Generation and analysis of random point mutations in an antibody CDR2 sequence: many mutated antibodies lose their ability to bind antigen. J. Exp. Med. 176:855-866.

Chien, N. C., Roberts, V. A., Giusti, A. M., Scharff, M. D., and Getzoff, E. D. (1989) Significant structural and functional change of an antigen-binding site by a distant amino acid substitution: proposal of a structural mechanism. Proc. Natl. Acad. Sci. USA. 86:5532-5536.

Chothia, C. (1976) The nature of the accessible and buried surfaces in proteins. J. Mol. Biol. 105(1):1-12.

Claflin, J. L., and Rudikoff, S. (1977) Uniformity in a clonal repertoire: a case for a germline basis of antibody diversity. Cold Spring Harbor Quant. Biol. 41:725.

Clarke, S. H., Claflin, J. L., Potter, M., and Rudikoff, S. (1983) Polymorphisms in anti-phosphocholine antibodies reflecting evolution of immunoglobulin families. J. Exp. Med. 157(1):98-113.

Cosenza, H., and Kohler, H. (1972) Specific inhibition of plaque formation to phosphorylcholine by antibody. Science 176:1027

Giusti, A. M., Chien, N. C., Zack, D. J., Shin, S-U., and Scharff, M. D. (1987) Somatic diversification of S107 from an antiphosphocholine to an anti-DNA autoantibody is due to a single base change in its heavy chain variable region. Proc. Natl. Acad. Sci. USA 84:2926-2930.

Glockshuber, R., Stadlmuller, J., and Pluckthun, A. (1991) Mapping and modification of an antibody hapten binding site: a site-directed mutagenesis study of McPC603. Biochem. 30:3049-3054.

Guddat, L. W., Shan, L., Anchin, J. M., Linthicum, D. S., Edmundson, A. B. (1994) Local and transmitted conformational changes on complexation of an anti-sweetener Fab. J. Mol. Biol. 236(1):247-74.

Ettinger, H. M., Julius, M. H., and Heusser, C. H. (1982) Mechanism of clonal dominance in the murine anti-phosphorylcholine response. I. Relation between antibody avidity and clonal dominance. J. Immunol. 128:1685-91

Herron, J. N., He, X. M., Mason, M. L., Voss, E. W., and Edmundson, A. B. (1989) Three-dimensional structure of a fluorescein-Fab complex crystallized in 2-methyl-2,4,-pentanediol. Proteins 5:271

Hudson, N. W., Bruccoleri, R. E., Steinrauf, L. K., Hamilton, J. A., Mudgett-Hunter, M., Margolies, M. N. (1990) A V kappa-J kappa junctional change in an antidigoxin recombinant antibody destroys digoxin-binding activity. J. Immunol. 145(8):2718-24.

Jeske, D. J., Jarvis, J., Milstein C., and Capra, J. D. (1984) Junctional diversity is essential to antibody activity. J. Immunol. 133(3):1090-2.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) Sequences of Prtoteins of Immunological Interest., 5th Edition, US Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH Publication No. 91-3242).

Kobrin, B. J., Buhl, S., Shulman, M. J., and Scharff, M. D. (1991) A V region mutation in a phosphocholine-binding monoclonal antibody results in loss of antigen binding. J. Immunol. 146(6):2017-2020.

Lotscher, M., Amstutz, H., Heusser, C. H., and Blaser, K. (1990) Fine specificity and heavy chain V gege usage of antibodies to the phosphorylcholine hapten. Mol. Immunol. 27(4):369-378.

Lotscher, M., Heusser, C. H., Amstutz, H., and Blaser, K. (1993) Fine specificity and VJ usage of light chains in antibodies to the phosporylcholine hapten. Eur. J. Immunol. 23:124-130.

Malipiero, U. V., Levy, N. S., and Gearhart, P. J. (1987) Somatic mutation in anti-phosphorylcholine antibodies. Immunol. Rev. 96:59-74.

Manser, T., Parhami-Seren, B., Margolies, M. N., and Gefter, M. L. (1987) Somatically mutated forms of a major anti-p-azophenylarsonate antibody variable region with drastically reduced affinity for p-azophenylarsonate, by-products of an antigen-driven immune response? J. Exp. Med. 166:1456-1463.

Morrison, S. L., Johnson, M. J., Herzenberg, L. A., and Oi, V. T. (1984) Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains. Proc. Natl. Acad. Sci. USA. 81:6851

Padlan, E. A., Davies, D. R., Rudikoff, S., and Potter, M. (1976) Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. Immunochem. 13:945-949.

Padlan, E. (1994) Anatomy of the antibody molecule. Mol. Immunol. 31(3):169-217.

Padlan, E. A., Cohen, G. H., and Davies, D. R. (1985) On the specificity of antibody/antigen interactions: phosphochoine binding to McPC603 and the correlation of three-dimensional structure and sequence data. Ann. Inst. Pasteur. Immunol. 136C(2) 271-276.

Pawlita, M., Potter, M., and Rudikoff, S. (1982) κ-chain restriction in anti-galactan antibodies. J. Immunol. 129(2):615-618.

Perlmutter, R. M. (1984) The molecular geneticsof phosphocholine-binding antibodies. In: The Biology of Idiotypes. Plenium Press, New York, pp. 59.

Quintans, J., and Cosenza, H. (1976) Antibody response to phosphorylcholine in vitro. II Analysis of T-dependent and T-independent responses. Eur. J. Immunol. 6:399

Roberts, V. A., Stewart, J., Benkovic, S. J., and Getzoff, E. D. (1994) Catalytic antibody model and mutagenesis implicate arginine in transition-state stabilization. J. Mol. Biol. 235(3):1098-116.

Rudikoff, S., Satow, Y., Padlan, E., Davies, D., and Potter, M. (1981) Kappa chain structure from a crystallized murine Fab': role of joining segment in hapten binding. Molec. Immunol. 18(8):705-11.

Satow, Y., Cohen, G. H., Padlan, E. A., and Davies, D. R. (1986) Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7 Å. J. Mol. Biol. 190:593-604.

Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., and Davies, D. R. (1974) The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. Proc. Natl. Acad. Sci. USA 71(11):4298-4302.

Sharon, J., Gefter, M. L., Wysocki, L. J., and Margolies, M. N. (1989) Recurrent somatic mutations in mouse antibodies to p-azophenylarsonate increase affinity for hapten. J. Immunol. 142:596.

Stenzel-Poore, M. P., Bruderer, U., and Rittenberg, M. B. (1988) The adaptive potential of the memory response: clonal recruitment and epitope recognition. Immunol. Rev. 105:113-136.

Stenzel-Poore, M. P., and Rittenberg, M. B. (1989) Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. J. Immunol. 143(12):4123-4133.

Stenzel-Poore, M. P., and Rittenberg, M. B. (1987) V gene diversity in the memory immune response to PC-KLH. In *H-2 Antigens*, (Edited by Chells, S. D.), pp. 827-835. Plenum Publishing Corporation. New York.

Stenzel-Poore, M. P., and Rittenberg, M. B. (1991) Unequal distribution of replacement mutations in kappa and lambda light chains and their associated heavy chains: the group II antibody response to phosphocholine-KLH. In *Somatic Hypermutation in V-Regions*, (Edited by Steele, E. J.), pp. 95-104. CRC Press.

Stewart, J. D., Roberts, V. A., Thomas, N. R., Getzoff, E. D., and Benkovic, S. J. (1994) Site-directed mutagenesis of a catalytic antibody: an arginine and a histidine residue play key roles. Biochem. 33(8):1994-2003.

Strickland, F. M., Gleason, J. T., and Cerny, J. (1987) Serological and molecular characterization of the T15 idiotype. I. Topological mapping of idiotopes on TEPC15. Mol. Immunol. 24:631.

Wicker, L. S., Guelde, G., Scher, I., and Kenny, J. (1982) Antibodies from the Lyb5 B cell subset predominate in the secondary IgtG response to phosphocholine. J. Immunol. 129:950-53

Zamyatnin, A. A. (1972) Protein volume in solution. Prog. Biophys. Mol. Biol. 24:107-23.