

DO ANTIBODIES RECOGNIZE AMINO ACID SIDE CHAINS OF PROTEIN ANTIGENS INDEPENDENTLY OF THE CARBON BACKBONE?

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An integral component within Jerne's idiotypic network theory (1) is the internal image antibody (Ab)¹ that bears determinants within the variable (V) region resembling external antigens. Internal images mimicking viruses, bacteria, protozoa, and natural ligands have been described (2-5) and have generated interest in their use as potential vaccines and probes for cellular receptors (6). The structural basis for internal image expression, however, remains largely unknown. Two recent studies using the synthetic (Glu⁶⁰Ala³⁰Tyr¹⁰)_n terpolymer (7) and the reovirus hemagglutinin (8) have identified determinants in Ab internal image V regions that appear to match linear or continuous sequences within each respective antigen. Further insight into the molecular nature of internal images and the rules that govern antigen recognition will be crucial for the rational design of internal image vaccines and an understanding of idiotypic interactions.

To investigate internal image expression in a well-defined protein system, we have used the a1 allotype of rabbit Ig as a model antigen (9-11) and have isolated two mouse mAbs that bear a1-like determinants within their antigen-binding sites (12). We have now obtained the V region amino acid sequences of these internal images and have found that both mAbs contain a unique sequence of identity with rabbit Ig but in a novel configuration. The data, coupled with results from synthetic peptide inhibition experiments, indicate that an amino acid sequence oriented in reverse direction on an internal image molecule can effectively mimic the nominal antigen.

Materials and Methods

Cell Lines. The hybridoma cell lines 6B12 and 61B8 have been described (12). They were derived from independent fusions of Sp2/0 cells (13) with spleen cells from A/J mice that had been hyperimmunized with rabbit anti-a1 Ab before fusion. Cultures of hybridoma cells

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¹ *Abbreviations used in this paper:* Ab, antibody; ACN, acetonitrile; FR, framework region; TFA, trifluoroacetic acid; V, variable region; CDR2, complementarity-determining region 2.

were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 15% (vol/vol) FCS (HyClone Laboratories, Logan, UT).

Primer Extension Sequencing. The cDNA sequences for each hybridoma were obtained using H and L chain mRNAs as templates for oligonucleotide primer extension, essentially as described by Caton et al. (14). Briefly, total cellular RNA was extracted from hybridoma cells by the guanidium isothiocyanate/cesium chloride method (15). After cell lysis and pelleting of the RNA through a CsCl gradient, the RNA pellet was further purified by two chloroform/butanol (4:1) extractions and repeated ethanol precipitations. After purification, the RNA was stored in 150- μ g aliquots under ethanol at -20°C .

Just before the preparation of full-length cDNA transcripts, the RNA was again precipitated and denatured with dimethylsulfoxide (Aldrich Chemical Co., Milwaukee, WI), and the pellets were washed with 70% (vol/vol) ethanol. Two oligonucleotide primers [5'-ATACAGTTGGTGCAGCATCAGCCCCG-3', specific for the constant region of κ L chain mRNA, and 5'-GGGGCCAGTGGATAGAC-3', specific for the constant region of IgG1, IgG2a, and IgG2b H chain mRNA] were 5' end-labeled with [γ - ^{32}P] ATP (ICN Biomedicals Inc., Irvine, CA) using T-4 cloned polynucleotide kinase (U.S. Biochemicals, Cleveland, OH). Mixtures of labeled primers, hybridoma RNA, reverse transcriptase (Life Sciences, St. Petersburg, FL), and deoxynucleotides were incubated for 2 h at 42°C and then treated with ribonuclease for 30 min. The cDNAs were extracted with chloroform/butanol (1:1), ethanol precipitated, and separated by electrophoresis in a 5% (wt/vol) acrylamide gel. After autoradiography, full-length cDNAs were isolated from the gel and sequenced by the Maxam and Gilbert procedure (16).

Three additional primers (specific for the V region of either the H or L chain) were also used for primer extension. These primers were based on the sequence generated using the constant region primers and are as follows: (a) 5'-TCCAATCCACTCAAGGCTCTT-3', specific for 6B12 V_H region; (b) 5'-ATAGATCAATAGTTTAGG-3', specific for 6B12 V_L region; and (c) 5'-GTGGATGCCAGTAGATCAG-3', specific for 61B8 V_L region.

Peptide Synthesis. The peptides used in this study were synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using phenylacetamidomethyl resin and *tert*-butyloxycarbonylamino acid derivatives. Peptides were analyzed and purified by reverse phase HPLC using the following conditions: Vydac C_{18} 300 \AA pore columns; flow rate 1 ml/min; buffer A, 0.1% (vol/vol) trifluoroacetic acid (TFA) in H_2O ; buffer B, 0.1% TFA in 90% (vol/vol) acetonitrile (ACN); gradient 0-100% buffer B in 30 min; detection at 214 nm.

Radioimmunoassay. Polyvinyl microtiter wells (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 μl of 100 $\mu\text{g/ml}$ affinity-purified 3-2F1, a mouse IgG1 mAb specific for the common a1 allotope (9). Unbound protein was recovered and the plates were washed three times with 0.01 M PBS, pH 7.0, containing 1% (wt/vol) BSA (Sigma Chemical Co., St. Louis, MO) and 0.05% (vol/vol) Tween 20 (Sigma Chemical Co.). Each peptide solution used as inhibitor was prepared by initially dissolving the peptide in 90% (vol/vol) ACN, 0.1% (vol/vol) TFA, followed by dilution of five parts peptide solution into 95 parts physiological saline (final concentration of peptide ranged from 3.5 mg/ml to 10 mg/ml in 4.5% [vol/vol] ACN, 0.005% [vol/vol] TFA). To each well, 50 μl of inhibitor were added and incubated for 24 h at 4°C . After incubation, 25 μl of ^{125}I -labeled a1 Ig (10^5 cpm; 30 ng) were added for 1 h at room temperature. The plates were washed 10 times with tap water and the amount of bound radiolabel was determined using a gamma counter (Packard Instrument Co. Inc., Downers Grove, IL).

Results

Expression of the a1 allotype on rabbit H chains has been correlated with two amino acids in framework region 1 (FR1; Arg-10 and Thr-13) and two amino acids in FR3 (Thr-84 and Glu-85) (17). We previously isolated from independent fusions two mAbs that demonstrate a1-like internal image activity, 6B12, an IgG2b κ mAb, and 61B8, an IgG1 κ mAb (12). To determine whether the V regions of these mAbs contained residues homologous to rabbit Ig, we performed Maxam-Gilbert sequencing

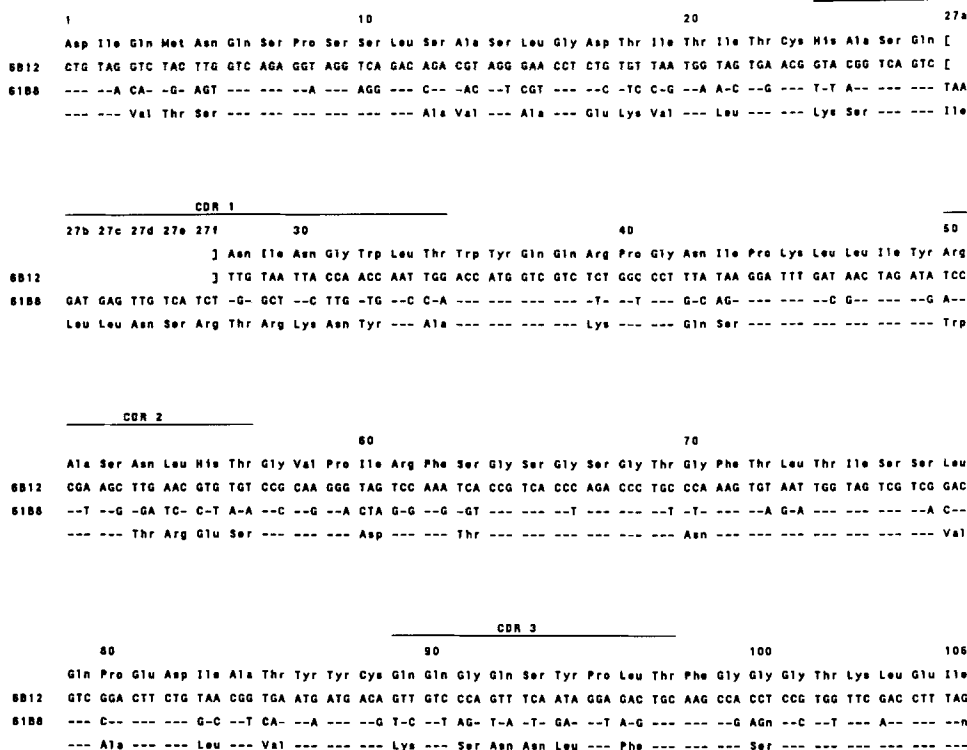


FIGURE 1. V region DNA and deduced amino acid sequences for 61B8 and 6B12 L chains. Amino acids are numbered from the amino to COOH termini following the scheme of Kabat et al. (24). Nucleotide sequences are given 3'→5'. Dashes (-) indicate identity at the nucleotide and amino acid level. Brackets ([]) indicate the absence of an insert in CDR1 of 6B12. Solid lines are included above each CDR. *n* = unknown nucleotide. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00807.

of full-length H and L chain cDNA transcripts that were prepared by reverse transcriptase primer extension of hybridoma RNA.

Internal Image L Chain Sequences. Partial V_L sequences for both internal image mAbs were obtained using a single primer complementary to the 5' constant region of κ L chain mRNA. The remaining V_L sequences were then determined using individual primers specific for either 6B12 or 61B8 (see Materials and Methods). The DNA sequences and the deduced amino acid sequences are shown in Fig. 1. The two mAb V_L regions were found to share only 62.2% amino acid sequence identity. An additional difference between the V_L regions was a six amino acid insert in 61B8 (positions 27a–27f) that was not present in 6B12. The degree of dissimilarity exhibited by the two L chains is consistent with the use of separate V_κ genes by each hybridoma. Most importantly, there were no apparent similarities between the L chain sequences of the mAbs and the $\alpha 1$ -associated residues of rabbit Ig.

Internal Image H Chain Sequences. The V region sequences for 6B12 and 61B8 H chains were obtained in a manner similar to those of the L chains. Although the

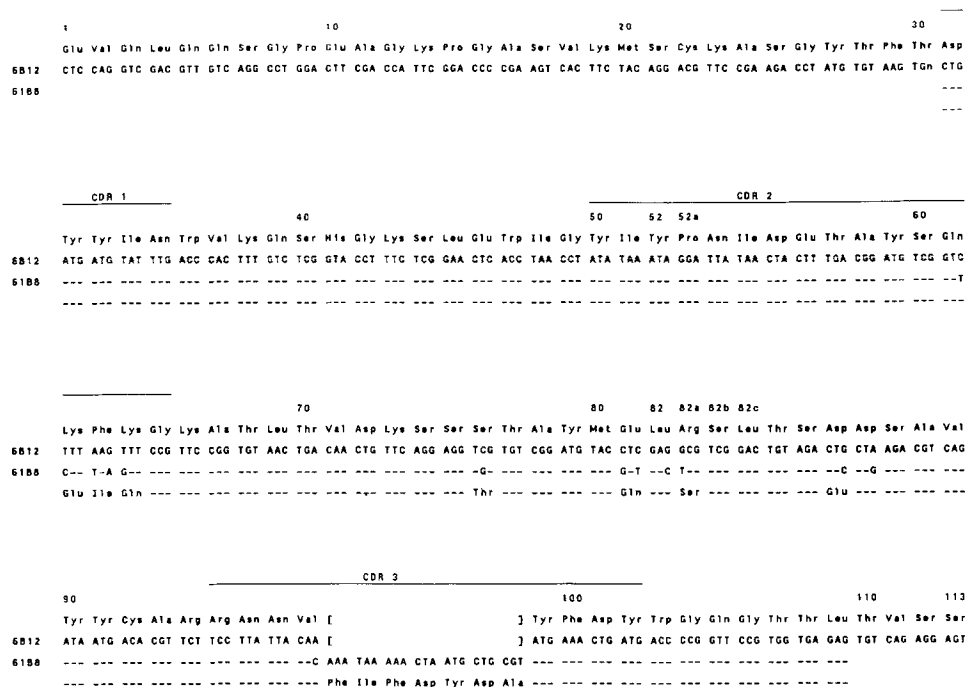


Figure 2. V region DNA and deduced amino acid sequences for 61B8 and 6B12 H chains. Amino acids are numbered from the amino to COOH termini following the scheme of Kabat et al. (24). Nucleotide sequences are given 3'→5'. Dashes (-) indicate identity at the nucleotide and amino acid level. Brackets ([]) indicate the absence of an insert in CDR3 of 6B12. Solid lines are included above each CDR. *n* = unknown nucleotide. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00807.

hybridomas were derived from separate mice and were of different H chain isotypes, the V_H DNA and deduced amino acid sequences showed a high degree of similarity, >95% identity at the DNA level and ~90% identity at the amino acid level (Fig. 2). Both H chains belong to the J558 V_H gene family (subgroup I) (18).

Interestingly, a sequence that included five amino acids with reverse homology to the rabbit a1 antigen was observed in complementarity-determining region 2 (CDR2) of each mAb V_H region (Fig. 3). The spacing of the conserved residues, though in opposite orientation, was identical to that seen in rabbit Ig. In addition, most of these amino acids occur only rarely in other known mouse sequences (18). Of significance was the presence of a paired Glu-56 and Thr-57 in both 6B12 and 61B8, the same amino acids in FR3 of rabbit Ig that correlate with a1 immunoreactivity (17). This pairing is unique among mouse sequences and only one other H chain within the J558 family possesses a glutamic acid at position 56 (18).

Peptide Inhibition of a1 Binding to Anti-a1. To directly test whether the reversed sequence of the mAb V_H region expressed an a1-like epitope, we used synthetic peptides to inhibit the binding of rabbit Ig to anti-a1 Ab. The first peptide, RA1, was a 15-mer that corresponded to the allotype-correlated residues in FR3 of rabbit a1

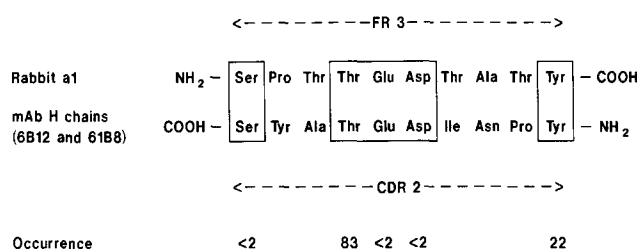


Figure 3. Region of observed homology between positions 81 and 90 of rabbit a1 FR3 and positions 52 and 60 of the mAb V_H CDR2. Occurrence refers to the observed frequency of that residue among sequenced members of the mouse J558 H chain family (%). Note that the rabbit a1 sequence is shown N→C while the mAb sequence is shown C→N.

Ig (positions 80–93). The second peptide, MA1, was a 15-mer homologous to 13 amino acids present in CDR2 and 2 amino acids present in FR2 of 6B12 and 61B8 (positions 48–61). The role of the internal image residues 56 and 57 in contributing to a1-like expression was assessed with the SMA1 peptide, identical to MA1 except for conservative substitutions at the two relevant positions (Glu→Asp and Thr→Ser). The UB1 peptide, bearing no homology to the above peptides, was used as a control. The peptides were tested for the ability to inhibit the binding of rabbit a1 Ig to an anti-a1 mAb, 3-2F1, which recognizes the common a1 allotope (9). The assay was performed by preincubating the inhibitors in Ab-coated wells for 24 h before addition of ¹²⁵I-a1 Ig.

It was found that peptides corresponding to either the rabbit a1 epitope or to the internal image CDR2 both competed for binding to Ab (Table I). In addition, the amounts needed for 50% inhibition did not differ significantly, indicating similar binding affinities. Neither the substituted peptide, SMA1, nor the control peptide, UB1, inhibited binding, providing evidence for the specificity of the reaction. Essentially identical results were obtained when the peptides were tested using polyclonal anti-a1 Ab (data not shown). Thus, MA1 and RA1, although composed of five homologous residues oriented in opposing directions, each appear to define an a1 epitope.

TABLE I
Peptide Inhibition of a1 Ig Binding to Anti-a1 Antibody

Inhibitor	Sequence	Required for 50% inhibition μM	Inhibition at 1 mM %
Rabbit a1 Ig	—	0.02	99
RA1 (rabbit a1 FR3)	I <u>TSPTTEDTATY</u> FCA	60	92
MA1 (Ab2 β CDR2)	I <u>GYIYPNIDETA</u> YSQ	100	90
SMA1 (substituted MA1)	I <u>GYIYPNID</u> DSAYSQ	>5,000	8
UB1 (control)	MQIFVKTLTGKDPGG	>5,000	0

Binding of ¹²⁵I-labeled rabbit a1 Ig to the anti-a1 mAb, 3-2F1, in the presence of rabbit Ig or synthetic peptides. All sequences are represented N→C and regions of reverse homology to RA1 are underlined. The RA1 peptide matches residues found within FR3 of rabbit a1 Ig that appear to determine allotype expression (positions 80–93) (17). MA1 represents 13 amino acids present in CDR2 and two amino acids in FR2 of 6B12 and 61B8 mAbs (positions 48–61). SMA1 is identical to MA1, except that the residues corresponding to CDR2 positions 56 and 57 are substituted. UB1 is a control peptide bearing no homology to RA1, MA1, or SMA1.

Discussion

Our results show that two internal image mAbs that mimic the rabbit a1 allotype contain unique V_H sequences homologous to the original antigen but in reverse orientation. Synthetic peptide inhibition experiments confirmed the ability of a reversed sequence to resemble the nominal antigenic epitope. In contrast to earlier studies in which internal image determinants were present in the expected orientation (7, 8), the results presented here suggest that the ability to simulate the structural motif of an antigen is dependent upon the configuration of amino acid side chains rather than the direction of the carbon backbone.

The rabbit a1 allotype, used in this study as a model antigen to investigate epitope structure and recognition, was found by synthetic peptide analysis to require a paired Thr and Glu in H chain FR3 for expression. The importance of these residues, as well as residues clustered in FR1, was previously suggested by examination of sequence data (17) and tryptic digests of rabbit H chains (19). In addition, computer-aided analysis of Ig structure has shown that the relevant positions in FR3 are exposed to solvent and accessible to a large surface probe (20). Thus, our results further support the concept that rabbit FR3 residues, including positions 84 and 85, encode the major a1 allotypic determinant. While the precise function of the FR1 amino acids remains unclear, it is possible that they are involved in expression of minor subpopulations of a1 Ig (21, 22).

The ability to recognize protein sequences in reverse orientation would serve to greatly increase the effective repertoire of the immune system. With regard to the idiotypic network, presentation of an epitope in an altered milieu could lead to imperfect mimicry of ligands; in the case of natural ligands, this could allow such images to be retained within the immune system yet not bind to endogenous receptors and thereby interfere with physiological functions. A perhaps analogous situation may exist at the level of antigen recognition by class II molecules in which it appears that an internal sequence is displaced by an imperfect image presented on an external antigen (23). Further studies to define the structural basis for internal image epitopes should lead to a better understanding of antigen recognition and to novel approaches for vaccine design.

Summary

In an effort to understand the structural basis for antigen mimicry by internal image antibodies, we determined the variable (V) region sequences of two mouse mAbs that mimic the rabbit Ig a1 allotype. The results showed that while the mAb light chains did not contain any allotype-related residues, both heavy chain V regions contained within complementarity-determining region 2 an unusual sequence homologous to the nominal antigen but in opposite orientation with respect to the carbon backbone. The ability of the internal image reversed sequence to express an a1-like determinant was tested directly by producing synthetic peptides that corresponded to the presumed antigenic regions of rabbit Ig and the mAb internal images, respectively. Although the two peptides presented the homologous residues in opposite orientations, they both completely inhibited at similar concentrations the binding of rabbit Ig to anti-a1 antibody. Conservative substitutions in the peptide sequence identified a paired Thr and Glu as being critical for expression of the a1 epitope. These findings

indicate that antibodies can recognize the molecular environments created by amino acid side chains independently from the orientation of the protein carbon backbone.

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